The Effect of Corticotropin-Releasing Hormone (CRH) on Gonadotropin-Releasing Hormone (GnRH) Neurons in Female Mice

by

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Dedication

To my mom, dad, sisters, and my beloved Cartoon

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ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	D-2-amino-5-phosphonovaleric acid
ARC	arcuate nucleus of the hypothalamus
AVP	arginine vasopressin
AVPV	anteroventral periventricular nucleus
Cm	capacitance
CNO	clozapine-N-oxide
CNQX	6-cyano-7-nitroquinoxaline
CORT	corticosterone
CRH	corticotropin-releasing hormone
CRHR	corticotropin-releasing hormone receptor
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DREADDs	designer receptor exclusively activated by designer drugs
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FSH	follicle-stimulating hormone
FWHM	full width at half maximum

GABA	gamma-aminobutyric acid
$GABA_A$	A-type gamma-aminobutyric acid receptor
GFP	green fluorescent protein
GnIH	gonadotropin-inhibitory hormone
GnRH	gonadotropin-releasing hormone
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
I_A	A-type potassium current
I_K	delayed rectifier potassium current
I_{hold}	holding current
ICV	intracerebroventricular
KNDy	arcuate neurons co-expressing kisspeptin, neurokinin B and dynorphin
LH	luteinizing hormone
MUA	multiunit activity
NMDA	N-methyl-D-aspartate
OVX	ovariectomized
OVX+E	ovariectomized and implanted with estradiol capsule
POA	preoptic area
PSC	postsynaptic current
PVN	paraventricular nucleus of the hypothalamus
Rin	input resistance
Rs	series resistance
TTX	tetrodotoxin
Ucn II	urocortin II
Ucn III	urocortin III

Abstract

Reproductive function is regulated through the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) neurons form final commons pathway that integrates inputs and releases GnRH, which then stimulates downstream elements of the HPG axis. Several factors can alter GnRH neuron output and thus reproductive function. One such signal is stress. Stress activates several pathways including the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) is released in response to activation of HPA axis and has been shown in several studies to be involved in stress-induced alteration of reproductive function. In this dissertation, our central hypothesis is that CRH acts on the HPG axis to alter GnRH neuron activity. To determine if CRH modifies firing activity of GnRH neurons, we performed extracellular recordings of GFP-identified GnRH neurons before and during CRH treatment. Several studies suggested that the interactions between HPA and HPG axes are modulated by estradiol. Therefore, GnRH neurons were recorded from ovariectomized mice either with an estradiol capsule implanted (OVX+E) or not treated further (OVX) to determine the influence of estradiol on the GnRH neuron response to CRH. In OVX mice, CRH did not affect GnRH neuron activity. In contrast, CRH displayed dose-dependent stimulatory and inhibitory effects on firing frequency of GnRH neurons in brain slices from OVX+E mice. This suggested that estradiol is required for CRH to alter GnRH neuron activity. The dose-dependent effects of CRH resulted from the activation of different CRH receptors. A CRH receptor (CRHR)-1 agonist increased firing activity in GnRH neurons, whereas activation of CRHR-2 led to decrease in firing frequency. We further analyzed if CRH alter short-term burst firing patterns, which has been associated with hormone secretion from neuroendocrine cells. We found that CRH affected burst frequency and other properties, including burst duration, spikes/burst, and/or intraburst interval. These results indicate that GnRH neuron activity is regulated by CRH. We further explored how CRH acts on GnRH neurons both through indirect

and direct mechanisms. First, we tested if CRH has an acute effect on activity of arcuate kisspeptin neurons, which provide estradiol-sensitive activation of GnRH neurons. CRH did not acutely alter firing rate of arcuate kisspeptin neurons in either OVX or OVX+E (both daily surge and low E models) mice. Second, we tested if GABAergic inputs, which is a major excitatory fast synaptic transmission to GnRH neurons, are influenced by CRH. Activation of CRHR-1 increased GABAergic postsynaptic current frequency in GnRH neurons from OVX+E, whereas activation of CRHR-2 showed no effect. This increase in GABAergic inputs was not observed in GnRH neurons from OVX mice treated with CRHR-1 agonist. This implies that CRH stimulate GnRH neuron activity by increasing GABAergic inputs to GnRH neurons and this effect is also estradiol-dependent. Third, we tested the direct action of CRH on GnRH neuron voltage-gated potassium currents, which play roles in regulating resting membrane potential and excitability of GnRH neurons. The amplitude of fast transient and sustained potassium currents in GnRH neurons from OVX+E was not affected by either activation of CRHR-1 or CRHR-2. Fourth, the direct action of CRH on GnRH neuron excitability was tested. No differences were observed in action potential firing in response to current injection from GnRH neurons treated with CRH. Overall, these data suggest that CRH exerts both stimulatory and inhibitory on GnRH neuron firing activity and the CRH is likely act on GnRH neurons through indirect mechanisms.

Chapter 1: Introduction

Overview

Reproduction is required to maintain species. In vertebrates, reproduction is controlled by the hypothalamo-pituitary-gonadal (HPG) axis. This axis is regulated by a number of external and internal inputs, such as season, food availability, age, and stress. The last of these is regulated by another neuroendocrine axis, the hypothalamo-pituitary-adrenal (HPA) axis, along with other central and peripheral elements. This introduction will focus on how different levels of the HPG and HPA axes interact, and how stress affects reproduction, in particular at the central level.

The HPG axis

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway that integrates inputs and releases hormone to control reproductive processes in vertebrates. The somata of GnRH neurons are located in the midventral preoptic area (POA) and hypothalamus and most project to the median eminence where GnRH is released near the primary capillaries of the hypophyseal portal system (1). There are two modes of GnRH release, pulse and surge. Pulsatile release of GnRH occurs during the majority of the female reproductive cycle and is the only known mode of release in males (2,3). At the end of follicular phase in females, GnRH release switches from pulsatile to surge mode, which in most species is critical for ovulation (4-7). GnRH binds to receptors on gonadotropes in the anterior pituitary gland, where it stimulates

synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from these cells (8-10). LH and FSH activate gonadal cells, regulating gametogenesis and steroidogenesis. Gonadal steroids feed back to the brain and pituitary to regulate GnRH and gonadotropin secretion, respectively (11-14).

There are two modes of release for GnRH, pulse and surge. Pulsatile release of GnRH occurs during the majority of female reproductive cycle and is the only mode of release in males. Daily measurement of LH showed that the LH level is fairly constant throughout the menstrual cycle with the exception of a preovulatory peak of LH, called a surge (15). With the development of radioimmunoassays it became possible to assay more frequent blood samples. Sampling at 10-min intervals from ovariectomized (OVX) monkeys revealed a pulsatile pattern with intervals between subsequent LH peaks around 60 minutes (16). Frequent sampling from pituitary portal blood in ewes revealed the LH pulse pattern mirrored that of GnRH (2,17). The pulsatile secretion of GnRH is necessary to maintain release of LH and FSH as shown by Belchetz and colleagues in 1978. In this study, endogenous release of GnRH was eliminated by ablation of the medial basal hypothalamus and GnRH was administered in a pulsatile or continuous manner. Continuous treatment with GnRH inhibited both LH and FSH secretion whereas pulsatile treatment of GnRH restored release of the gonadotropins (18). This emphasizes the importance of GnRH release pattern for reproductive function.

The frequency of GnRH release varies during the cycle with the changes in sex steroid hormones. Low level of estradiol during the majority of the cycle exerts negative

feedback effect on GnRH pules. The rise in estradiol levels as the ovarian cycle proceeds decreases GnRH/LH pulse amplitude but increases GnRH/LH pulse frequency (4,19). Estradiol also exerts an inhibitory effect at the pituitary level by suppressing response to GnRH stimulus in gonadotrophs (20,21). At the end of follicular phase, exposure to sustained high levels of estradiol initiates a switch from negative to positive feedback action and induces a GnRH surge during which release is continuous for many hours (4-6,22). The surge of GnRH induces LH surge that triggers ovulation (4-6,22). In primates, however, a surge of GnRH is not always required for the LH surge to occur. Exogenous treatment with pulsatile GnRH in monkeys with hypothalamic lesions or women with primary amenorrhea restored the menstrual cycle and ovulation; this is likely attributable to estradiol-mediated increases in response to GnRH at the pituitary being sufficient to generate a preovulatory surge (23,24). After ovulation, a corpus luteum is formed from remaining follicular tissues, marking the beginning of the luteal phase of the cycle. During this phase, high progesterone has negative feedback effects on GnRH pulse frequency (25,26). Progesterone and estradiol act together to suppress GnRH pulse frequency; the inhibitory effect of progesterone is estradiol-dependent as expression of the progesterone receptor requires estradiol (26-30). In the absence of pregnancy, the corpus luteum is degraded, decreasing circulating progesterone and its negative feedback action, leading to an acceleration of pulsatile release and the start of a new follicular phase.

Stress and the HPA axis

In 1936, Hans Selye first defined ‘general adaptation syndrome’ (31), which was later renamed ‘stress’. General adaptation syndrome referred to ‘the non-specific neuroendocrine response of the body’ to a noxious insult (32). Stress activates several pathways to help maintain homeostasis in these circumstances. The major pathways are a noradrenergic pathway in the locus coeruleus, epinephrine release from the adrenal medulla, and the hypothalamo-pituitary-adrenal (HPA) axis, which regulates the synthesis of glucocorticoids (reviewed in (33,34)). This study will focus primarily on the relationship between the HPA and HPG axes.

Corticotropin-releasing hormone (CRH), a 41-amino-acid peptide sequenced in 1981, plays a major role in the stress response (35). CRH regulates the HPA axis and is synthesized and released from neurons in the parvocellular subdivision of the paraventricular nucleus (PVN) of the hypothalamus. Of note, CRH is also expressed in other parts of the brain, such as the central nucleus of amygdala and hindbrain regions, which control the autonomic response to stress (36). Arginine vasopressin (AVP) is also synthesized in the parvocellular PVN and can augment the response to CRH (37). Magnocellular neurons in PVN and supraoptic nucleus also release AVP, typically in response increase in blood osmolarity (38); this source does not appear to be involved in the stress response, likely because CRH is not being co-released. CRH acts on pituitary corticotropes to stimulate secretion of adrenocorticotropic hormone (ACTH) (39,40), which ultimately triggers the synthesis of glucocorticoids (mainly corticosterone/cortisol) by the adrenal cortex (detailed review in (41)). Glucocorticoids

exert negative feedback effects on the pituitary and hypothalamus to regulate the activity of the HPA axis (42).

CRH receptors and other ligands

CRH receptors are class-B G-protein-coupled receptors (43,44). There are two major types of CRH receptors (CRHR), CRHR-1 and CRHR-2, which share 70% amino acid homology (45). The distribution of these two receptors is different. CRHR-1 is expressed primarily and widely in the brain, in regions including the cerebral cortex, amygdala, hippocampus, and hypothalamus, as well as in pituitary corticotropes (45,46). CRHR-2 is expressed mostly in peripheral tissues including heart and skeletal muscle, as well as in the lateral septum and hypothalamus (45,46). In addition to CRH, these receptors also bind a family of three CRH-related peptides called urocortins (47,48). CRH and the urocortins bind to CRHRs with different affinities. CRHR-1 has a higher affinity for CRH than CRHR-2 (IC_{50} = CRHR-1 1.6nM, CRHR-2 42nM) (49), but both receptors bind urocortin I with similar, and higher, affinity (IC_{50} = CRHR-1 0.16nM, CRHR-2 0.86nM) (49). Both urocortin II and urocortin III are considered to be endogenous ligands of CRHR2 due to the high affinity of CRHR-2 for these two peptides. Urocortin II and II have been used as tools to test independent activation of this subtype as CRHR-1 has little to no affinity for these peptides (48). The endogenous role of urocortins with regard to the regulation of HPA axis has been discussed in (50).

The HPA axis response to stress

When stress occurs, CRH is released near the hypophyseal portal vessels, and binds to CRHR-1 on corticotropes to drive downstream activity of the HPA axis. The role of CRHR-1 in the stress response was confirmed in CRHR-1-deficient mice, which exhibit an impaired stress response with minimal ACTH release and little to no corticosterone production (51). These mice also have reduced anxiety-related behaviors (52,53). In contrast, mice that lack urocortin I, which also binds with CRHR-1, had a normal corticosterone rise in response to restraint stress (54), despite the demonstrated ability of this peptide to increase glucocorticoids when exogenously administered (47). These observations imply that CRH and not urocortin I is the primary endogenous regulator of the CRHR-1-mediated stress response of the HPA axis, although redundancy in this system cannot be excluded.

Compared to CRHR-1, the function of CRHR-2 in the stress response is more complex. One hypothesis is that CRHR-2 acts to counteract the functions of CRHR-1 by lessening the stress response (55). Consistent with this, mice lacking CRHR-2 exhibit hypersensitivity to stress induction and a slower return of corticosterone to pre-stress levels (56,57). These results suggest that CRHR-1 and CRHR-2 might be responsible for different physiological responses during stress. This is of interest to interactions between the HPA and HPG axes, because while stress has been reported to primarily inhibit various reproductive processes, activation has also been observed. Of note, intracerebroventricular (icv) or intravenous injection of urocortin III (which binds exclusively to CRHR-2) increased ACTH and corticosterone levels compared to the

control group (58), suggesting activation of CRHR-2 can also initiate HPA axis activity; whether or not this occurs during stress in response to endogenously released urocortin III is not known.

Inhibitory effects of stress on reproduction

Deleterious effects of stress exposure on reproductive function have been demonstrated in several species (59-62). Several modalities for inducing a stress response have been used, including metabolic, immunological, psychosocial, and physical; these stressors alter timing of puberty in both males and females (63-67), and disrupt reproductive cycles in female (68-73). A likely factor contributing to altered reproduction is an underlying disruption of gonadotropin secretion. In particular, the effects of stress or HPA hormones on LH release have been informative, as the frequency of pulses provides a window to the central aspects of HPG axis function. It is important to keep in mind that stress induction and components of the HPA axis (CRH, ACTH, and glucocorticoids) are not equivalent. Stress induction stimulates multiple pathways, which includes but is not limited to the HPA axis. Nonetheless, treatments blocking response to or mimicking action of different components of the HPA axis have been useful in identifying their roles in stress-induced changes.

Glucocorticoids, the end organ product of HPA axis activation, typically suppress gonadotropin secretion, but data vary, and several factors need to be considered when interpreting these results. In ovariectomized (OVX) ewes, cortisol treatment that mimics the levels achieved during immunological stress suppresses LH pulse amplitude but not

frequency (73,74); this same cortisol treatment had no effect on GnRH pulse amplitude or frequency (74), indicating cortisol acts at the pituitary in this model to inhibit the response to GnRH, rather than centrally to affect its release. In contrast, cortisol suppressed both GnRH and LH pulse frequency in follicular phase ewes (61,75,76). This suggests that circulating ovarian steroids present in the latter study may influence the response of the HPG axis during stress; this will be discussed in detail below. In OVX female rats with low physiological estradiol replacement resembling diestrous levels, neither acute nor chronic treatment with corticosterone altered LH pulse frequency or amplitude (65,77), whereas in intact male rats chronic corticosterone treatment lowered serum LH levels in single terminal samples (78). The difference in LH response between these studies may be attributable to sex, corticosterone dose, which was lower in the former, and/or LH sampling regimen. In castrated male monkeys, chronic cortisol treatment suppressed LH and FSH in daily or alternate daily samples (79). This suppression is likely at the hypothalamic level because pituitary response to GnRH treatment was not affected. In early-follicular-phase women, chronic hydrocortisone treatment suppressed LH pulse frequency (62), whereas acute treatment on the day of sampling did not affect either LH or FSH levels in either early follicular phase women or in men (80). Here the discrepancy in results might be due to treatment duration or to different blood sampling intervals affecting the ability to detect LH pulses. Glucocorticoids thus suppress LH secretion in most conditions but the mechanisms (central vs pituitary) depend on the species, duration of treatment, and gonadal steroids.

The effects of CRH on gonadotropes and GnRH neuron output also vary with model studied. Inhibitory effects of CRH administered ICV or into the median eminence on LH pulse frequency and amplitude have been observed in monkeys and rats (81-83). While a critical role of CRH signaling in this scenario is likely to induce a glucocorticoid rise, intravenous injection of CRH inhibited LH pulse frequency and amplitude in adrenalectomized monkeys despite these animals being unable to produce adrenal glucocorticoids (83). This indicates that CRH itself can act on the reproductive system independent of glucocorticoids. CRH mediates stress effects on LH secretion, at least in part, as pretreatment with non-specific CRHR antagonists prevents stress-induced suppression of LH pulses in castrated male rats, OVX+E rats, and OVX monkeys (60,81,84,85). *In vivo* treatment of OVX+E rats with CRHR-1- or CRHR-2-specific antagonists revealed that different types of stressors act on the reproductive system via different CRHR subtypes; insulin-induced hypoglycemia and immunological stress act through CRHR-2, whereas restraint-induced stress occurs through both CRHR-1 and CRHR-2 (85). Administration of a CRHR-2 antagonist, astressin-2B, ameliorated the effect of restraint stress on LH pulse suppression but not the stress-induced corticosterone rise (86). In a complementary study, ICV administration of the CRHR-2-specific agonist urocortin II suppressed LH pulse frequency, but this effect was delayed by over an hour compared to the effect of stress, suggesting other systems activated by stress have a more rapid effect. An anti-urocortin II antibody blocked the inhibitory effect of restraint stress on LH in single point samples but did not alter either ACTH or corticosterone levels (87), suggesting that urocortin II could act at the central level.

Stimulatory effects of stress on reproduction

Stress exerts stimulatory effects on the reproductive system in some conditions. Several lines of evidence indicate CRH itself may activate the HPG axis. First, ICV administration of CRH in either male or female gonadectomized steroid-replaced sheep increased LH pulse frequency (88). Second, pretreatment with a CRHR antagonist that blocks both CRHR-1 and CRHR-2 prevented the stimulatory effect of interleukin-1 (IL-1), which mimics immune-mediated stress, on LH secretion in OVX+E monkeys (89). Third, pretreatment with a CRHR-1, but not CRHR-2, antagonist abolished the stimulatory effect of acute restraint stress on LH pulses in female rats, implying different consequences of activating these receptors (90,91). Fourth, in male rats, the initial exposure to restraint stress increased plasma LH levels within 15-30 min of stress onset, after which levels declined toward pre-stress baseline (92). This acute increase in LH secretion disappeared after 10 days of exposure to the same stress, suggesting a change in the response to stress over time (92). Together these observations suggest that under certain conditions, CRH can enhance LH secretion via CRHR-1.

Stimulatory effects of stress sometimes occur in a subset of animals. For example, examination of the individual rat data in (86) revealed that restraint stress during CRHR-2 antagonist treatment caused a transient increase in LH pulse amplitude in the representative data shown; whether or not this was a consistent observation is not known. Similarly, one of four monkeys subjected to 6h restraint showed increased LH pulse frequency and amplitude within 30 min of stress onset during both follicular and luteal phases (93). Intravenous injection of CRH caused premature hypothalamic

multiunit activity (MUA) volleys in 5 of 15 OVX monkeys tested (82); these data were noted to be excluded by the authors despite one third of animals exhibiting this response. If such observations are regarded as technical errors and excluded from the analysis, this could alter interpretation of the complete effects of stress on reproduction, in particular variations between stress-susceptible and stress-resilient subpopulations. These data point to potential biphasic effects of stress on reproductive neuroendocrine function with an initial stimulation followed by suppression.

Influence of sex steroid hormones on stress response

It is well established that sex differences exist in HPA axis function both under unstressed control conditions and during the stress response. Female rats produce both greater basal levels and greater increases in ACTH and corticosterone in response to stress than males (94,95). There are several factors that may underlie these sex differences, including gonadal steroid hormones. In this regard, estradiol can alter the function of the HPA axis at every level. CRH neurons express ERß (96,97) thus can respond directly to estradiol as well as be influenced via steroid-sensitive afferents. During unstressed conditions, rats with high circulating estradiol (OVX+E and proestrus) had higher basal ACTH and corticosterone levels (98,99); the corticosterone rise in response to stress was also greater in these animals compared to OVX or diestrous rats (98,100). CRH mRNA expression in the PVN is higher in OVX+E than OVX rats (77,99); further, basal CRHR-1 and CRHR-2 mRNA expression in the dorsal raphe was also higher in female than male rats (101). Stress-induced expression of CRHR-1 was greater during the morning of proestrus than diestrus, and removal of ovaries decreased

CRHR-1 and CRHR-2 mRNA expression in female rats, suggesting estradiol plays a role in regulation of CRHR expression (102,103). Both CRH and CRHR genes contain estrogen-responsive elements (104,105). Estradiol-induced increases in CRH and CRHR could thus prime the central arm of the HPA axis to produce a greater output in response to stress, and upregulate target tissue response, respectively.

In contrast to estradiol, androgens typically suppress the stress response. Castrated males produce a greater corticosterone rise in response to stress than intact males in several species (94,106,107). Androgen replacement by dihydrotestosterone (DHT), a non-aromatizable androgen, restored the corticosterone rise to that observed in intact male rats (107). The increase in corticosterone secretion observed in castrated males might be attributable to central effects of steroids as castrated rats have increased basal hypothalamic tissue CRH levels and increased CRH immunoreactivity in the PVN compared to intact rats (108). DHT replacement reduced stress-induced activation of neuroendocrine cells detected by cfos expression in the PVN (intact males were not examined) and also suppressed CRH and AVP mRNA expression in the PVN compared to unreplaced castrated rats (109). Together, these results indicate that androgens often play an opposite role to estrogens and that sex steroids are an underlying cause in sex differences in the stress response.

The effects of stress on the reproductive system are also influenced by gonadal steroid hormones. In rats, the inhibitory effects of insulin-induced hypoglycemia and food deprivation on LH pulse frequency were stronger in OVX+E than OVX rats

(77,110,111). Immunological challenge with IL-1 in female monkeys during the mid-follicular phase generated a robust increase in the mean circulating LH concentration five hours after injection, whereas similar treatment in early follicular phase monkeys, which have lower estradiol, had no effect, and the same treatment in OVX monkeys suppressed LH (112-114). In postmenopausal women receiving transdermal estradiol replacement, inflammatory stress or ACTH infusion increased mean hourly plasma LH concentrations 3-fold compared to baseline (115). Together these observations suggest gonadal steroids might dictate the direction of the effects of immune stress on gonadotropin secretion.

Estradiol may augment effects of stress by amplifying the actions of CRH. OVX+E rats exhibit a stronger suppressive effect of CRH on LH pulse amplitude and frequency than OVX rats (81). The inhibitory effects of CRH on GnRH mRNA expression in GnRH-producing immortalized hypothalamic GT1-7 cells is greater with estradiol co-treatment (116). The inhibitory effect of corticosterone is also influenced by estradiol as chronic corticosterone treatment suppressed LH pulse frequency and amplitude and decreased cfos expression (as a marker of neural activation) in arcuate kisspeptin neurons in OVX+E but not in OVX mice (117). Together, these data suggest that estradiol potentiates the effects of stress on the reproductive system, indicating bidirectional interactions between the HPG and HPA axes.

Effects of stress on the GnRH/LH surge

In addition to disruptions of homeostatic feedback, stress can interfere with estradiol positive feedback needed to generate ovulation. In rats, restraint for 5-7 hours starting 0-2 hours before the typical onset of the LH surge blocked the surge in half of the animals and decreased ovulation rate (118). In mice, the LH surge was blocked in a majority of animals in response to application of an acute psychosocial stress paradigm on the morning of proestrus. An LH surge was also not detected on the next day suggesting that stress did not delay LH surge by 24h, but either completely blocked the generation of the LH surge or altered its timing sufficiently to be out of the sampling window (119). That this stress-induced effect was on estradiol action, not production of the estradiol rise, is demonstrated by two observations. First, uteri of stressed mice were similar size to controls and uterine mass is directly correlated with estradiol levels (120). Second, the estradiol-induced LH surge was also blocked.

Glucocorticoids released during the stress response may inhibit the LH surge. Cortisol infusion in ewes during the early-to-mid follicular phase blocked the LH surge in half of the animals and delayed the surge in the remainder (75). The effects on the LH surge might result from the reduced or absent follicular phase estradiol rise attributable to suppressed pulsatile release (75). Further experiments, however, showed suppressive effects of cortisol on the estradiol-induced LH surge in both sheep and mice (69,121,122), indicating an additional effect of glucocorticoids to interrupt the positive feedback effects of estradiol. CRH might also be involved in stress-induced suppression of the LH surge. Continuous infusion of CRH for six hours starting an hour before the

typical onset of the LH surge or a single injection of CRH five minutes before typical surge onset reduced LH surge amplitude in proestrous rats (123). CRH-deficient mice, however, showed typical suppression of the LH surge by restraint stress (124), suggesting CRH might not be the only contributor in the stress-induced suppression of LH.

Possible central mechanisms of stress-reproductive interactions

GnRH neurons

Several studies suggest that GnRH neurons could be a direct target for CRH. Anatomical evidence showed that CRH-containing fibers in humans (125) and rats appose GnRH neuron cell bodies (126). To ask if effects of CRH may be direct on GnRH neurons, several labs have examined CRHR expression in these cells, but results are mixed. Immortalized GT1-7 cells express CRHR-2 and this expression is enhanced by estradiol (116). Single-cell RT-PCR analysis showed CRHR-1 mRNA in one-fourth of GnRH neurons from female mice and approximately 30% of GnRH neurons exhibited CRHR immunoreactivity although the antibody used did not distinguish between CRHR subtypes (127). Neither CRHR-1 nor CRHR-2 mRNA were enriched in GnRH neurons from male (intact or castrated) or female (diestrous or OVX) mice determined by translating ribosome affinity purification (128); lack of enrichment does not indicate absence and this method has reduced sensitivity with low copy-number transcripts such as GPCRs. Of note, CRHRs were not detected in cells identified as GnRH neurons from male and female mice determined by the single-cell RNA-seq transcriptional profiling but this approach may lack the necessary sequencing depth (129). *In situ* hybridization in female rats and male mice also did not detect either

CRHR-1 or CRHR-2 in GnRH neurons (130,131). These discrepancies might result from the species, sex, and methodical differences in each study. Cholera toxin injected into the third ventricle labels GnRH neurons among many cell types, but none of these labeled cells projected to CRH neurons (130). Together these results indicate expression of CRHR by GnRH neurons remains controversial. Even if CRHR is confirmed in GnRH neurons, the effects of stress on these cells can also be through intermediate neurons and/or through non-HPA stress pathways.

Despite the difficulties in confirming CRHR expression in GnRH neurons, functional studies suggest that GnRH neurons are direct and/or indirect targets for stress. Follicular phase monkeys that were classified as stress-sensitive (exhibited suppression of menstrual cycles and sex steroid levels during the first cycle of stress exposure) had less GnRH fiber immunostaining in the median eminence, but a higher number of neurons expressing GnRH mRNA in the medial basal hypothalamus, compared to monkeys that were resistant to stressors (continued to show normal menstrual cycles and changes in sex steroids) (132). This suggests that stress could alter transportation of GnRH from cell bodies to axon terminals, thus decreasing the releasable pool of GnRH. CRH acutely suppressed hypothalamic MUA volleys concomitant with the suppression of LH pulse frequency in two-thirds of monkeys tested (82), indicating an inhibitory action of CRH at the hypothalamic level.

Kisspeptin neurons

GnRH neuron activity and the response to stress are regulated by feedback signaling from sex steroid hormones. Since GnRH neurons do not express detectable levels of most steroid receptors (133-136), it is likely that sex steroid feedback is mediated by steroid-sensitive afferent neurons; steroid modulation of the stress response likely also occurs via afferents. Kisspeptin neurons are a major candidate as they express estrogen receptor α (ERα) (137) and play a role in negative and positive estradiol feedback on the HPG axis (138,139). Kisspeptin is a potent activator of GnRH neuron activity, and GnRH and gonadotropin release (140-143). In rodents, kisspeptin neurons are found in two major regions in the hypothalamus: the anteroventral periventricular (AVPV) nucleus and the arcuate nucleus (144).

With regard to the stress response, immunological and metabolic stresses decrease *Kiss1* and *Kiss1r* expression in hypothalamus of male mice and female rats (65,145-147). Although acute restraint stress did not change the number of *Kiss1*-expressing cells in the arcuate, activation of arcuate kisspeptin neurons as measured by cfos expression decreased after restraint stress in both male and female mice (148,149). This suggests that multiple aspects of kisspeptin neurons could be targeted by stress depending on the paradigm used. The effect of stress on *Kiss1* expression might be mediated through CRH and/or corticosterone as administration of either substance had similar inhibitory effects on hypothalamic *Kiss1* expression (65). One immunofluorescence study showed that both AVPV and arcuate kisspeptin neurons express CRHR (subtype not identified) but that only AVPV kisspeptin neurons express

glucocorticoid receptors (150). Another study examined expression of GFP driven by the CRHR-1 promoter and indicated expression of CRHR-1 in AVPV kisspeptin neurons (151), but the expression of CRHR in arcuate kisspeptin neurons awaits confirmation. Retrograde viral tracing failed to identify CRH neurons in the PVN as afferents of arcuate kisspeptin neurons (152). Further studies are needed to determine if arcuate kisspeptin neuron activity is regulated by CRH and other systems activated by stress.

GABAergic signaling

Gamma-aminobutyric acid (GABA) is one of the major regulators of GnRH neurons. In contrast to most neurons, GABA is typically an excitatory input to GnRH neurons due to the high intracellular concentration of Cl^- in these cells (153,154). GABAergic inputs to GnRH neurons are modified by several factors including pubertal developmental, steroids, and metabolic status (155-158). Various stressors increased activity of GABAergic neurons detected by cfos expression in the medial preoptic area (mPOA) (159-161), where GnRH neurons reside, but whether or not these are direct afferents of GnRH neurons is not known. Pretreatment with $GABA_A$ or $GABA_B$ antagonists in the mPOA or arcuate nucleus prevented the inhibitory effect of restraint or immunological stress, and CRH treatment on LH pulse frequency (162,163). This indicates that GABAergic signaling is necessary for stress or CRH to alter LH secretion. Moreover, administration of CRH to the bed nucleus of the stria terminalis or locus coeruleus increased neuronal activity measured by cfos expression in GAD67-identified GABAergic neurons in the mPOA (164,165). Therefore, it is possible that stress modulates GABAergic signaling to GnRH neurons via CRH signaling.

Gonadotropin-inhibitory hormone (GnIH) neurons

The discovery of an inhibitory regulator of GnRH neurons in 2000 by Tsutsui and colleagues identified another pathway involved in regulating the HPG axis (166). GnIH was first discovered in birds and named based on its physiological function. GnIH was later identified in other species; because it shares a structure of LPXRFamide (where X is L or Q), GnIH is known as RFamide-related peptide-3 (RFRP-3) in mammals. GnIH neurons project to GnRH neurons and inhibit activity of GnRH neurons via GnIH receptors (167,168). In addition to possible direct action at GnRH cell bodies, GnIH neurons also project to the median eminence and GnIH inhibits gonadotropin secretion from the pituitary (169). GnIH neurons express ERα and androgen receptors in female and male hamsters, respectively (168), thus can respond directly to sex steroids.

Recently, GnIH was shown to be a possible link between the HPA and HPG axes. The first evidence that supported this hypothesis was that GnIH expression is increased by stress in sparrows (170). Later, acute and chronic immobilization stress were shown to upregulate GnIH expression in the dorsomedial hypothalamic area in male rats (171). Further, GnIH expression was negatively correlated to plasma LH concentration. Stress also increases cfos expression in GnIH neurons in male and female mice and in ewes (148,149,172). Together, these data support the hypothesis that GnIH can mediate stress-induced inhibition of LH release. It is important to note that metabolic stress did not affect GnIH expression or activation of GnIH neurons (173). Therefore, GnIH might mediate some but not all inhibitory effect of stress on the HPG axis. Stress might act on

GnIH neurons via CRH and/or corticosterone due to the presence of both CRHR and glucocorticoid receptors in these cells (171). However, physiological studies on the mechanisms of stress and GnIH are still needed.

Dissertation preview

Prior to the studies in this dissertation, research in the field of stress and reproduction focused on the effects of stress on gene/protein expression or the GnRH/LH release. Our main goal is to study the mechanisms of how stress acts via CRH peptide on central components of HPG axis. Chapter 2 focuses on the effects of CRH on GnRH neuron firing activity and the influence of estradiol on the actions of CRH. Chapter 3 investigates the possible mechanisms of how CRH acts on GnRH neurons. This includes indirect mechanisms via neuromodulators and fast synaptic transmission to GnRH neurons and direct action on GnRH neurons. Chapter 4 summarizes the findings, discusses the results, proposes the future directions of the studies. Together, these studies provide the insight of how stress alters reproductive functions via the interactions between CRH and components of HPG axis.

Chapter 2: Estradiol-dependent Stimulation and Suppression of Gonadotropin-releasing Hormone (GnRH) Neuron Firing Activity by Corticotropin-releasing Hormone (CRH) in Female Mice

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Abstract

GnRH neurons are the final central regulators of reproduction, integrating various inputs that modulate fertility. Stress typically inhibits reproduction but can be stimulatory; stress effects can also be modulated by steroid milieu. CRH released during the stress response may suppress reproduction independent of downstream glucocorticoids. We hypothesized CRH suppresses fertility by decreasing GnRH neuron firing activity. To test this, mice were ovariectomized and either implanted with an estradiol capsule (OVX+E) or not treated further (OVX) to examine the influence of estradiol on GnRH neuron response to CRH. Targeted extracellular recordings were used to record firing activity from green fluorescent protein (GFP)-identified GnRH neurons in brain slices before and during CRH treatment; recordings were done in the afternoon when estradiol has a positive feedback effect to increase GnRH neuron firing. In OVX mice, CRH did not affect the firing rate of GnRH neurons. In contrast, CRH exhibited dose-dependent

stimulatory (30 nM) or inhibitory (100 nM) effects on GnRH neuron firing activity in OVX+E mice; both effects were reversible. The dose-dependent effects of CRH appear to result from activation of different receptor populations; a CRHR-1 agonist increased firing activity in GnRH neurons, whereas a CRHR-2 agonist decreased firing activity. CRH and specific agonists also differentially regulated short-term burst frequency and burst properties, including burst duration, spikes/burst and/or intraburst interval. These results indicate that CRH alters GnRH neuron activity and that estradiol is required for CRH to exert both stimulatory and inhibitory effects on GnRH neurons.

Precis

CRH exerts both stimulatory and inhibitory effects on GnRH neuron firing activity via activation of different receptors in an estradiol-dependent manner.

Introduction

GnRH neurons are crucial regulators of the reproductive system. GnRH pulses stimulate secretion of the gonadotropins, LH and FSH, which then induce the gonads to activate gametogenesis and steroidogenesis. GnRH release at the median eminence typically requires action potential firing (174). Many factors that alter GnRH neuron action potential firing regulate fertility (12,175,176). Stress is one of these factors. In some cases, acute stress can stimulate the reproductive system (90,177,178). The vast majority of studies, however, suggest that stress exposure suppresses reproductive function. Various types of stressors suppress LH pulses in several species, including rats (77,163), monkeys (93,179,180), sheep (181-183) and mice (149).

The hypothalamic-pituitary-adrenal (HPA) axis is one pathway that is activated in response to stress. Stress initiates hypothalamic release of corticotropin-releasing hormone (CRH), which stimulates secretion of ACTH from pituitary and thereby increased glucocorticoid production by the adrenal cortex. Activation of the HPA axis as monitored by increased circulating glucocorticoids during the stress response commonly occurs concomitantly with inhibition of the reproductive system (119,184,185). Some studies, however, suggest that CRH could have a direct effect on reproduction independent of the downstream glucocorticoid pathway. Pretreatment with CRH receptor antagonists prevents stress-induced suppression of LH pulses (81,84,85). In addition, intracerebroventricular or median eminence administration of CRH inhibits LH pulses (81-83) and hypothalamic multiunit electrical activity (MUA) volleys that are associated with LH pulses (82). This inhibitory effect of CRH on LH pulses persists in adrenalectomized monkeys, which lack the main glucocorticoid production site.

Interactions between the HPA axis and the reproductive system are modulated by estradiol. For example, estradiol potentiates inhibitory effects of insulin-induced hypoglycemia (77,111) and food deprivation (110) on LH pulses in ovariectomized (OVX) animals compared to those without estradiol treatment. OVX rats with estradiol replacement exhibit more severe suppression of LH secretion after CRH treatment than OVX rats (81). Further, estradiol treatment increases CRH receptor type-2 mRNA expression in immortalized GnRH neurons (GT1 cells) (116). These data indicate that

estradiol typically enhances the effect of stress on reproduction, potentially via a CRH-mediated pathway.

Anatomical observations suggest that CRH-producing neurons can directly affect GnRH neurons. CRH-containing fibers synapse on GnRH neurons (125,126). Approximately 30% of GnRH neurons in female mice exhibit CRH receptor immunoreactivity (127). The antibody used did not distinguish between CRH receptor subtypes, however in the same paper, single-cell gene expression profiling was positive for CRHR-1 but not CRHR-2 (127). Intracerebroventricular injection of CRH decreases GnRH mRNA expression in ewes (186), and studies in GT1 cells suggest the inhibitory effect of CRH on GnRH mRNA expression could be direct (116). These lines of evidence suggest CRH may alter GnRH neuron function as one mechanism of altering the reproductive system. Here, we examined the effect of CRH on GnRH neuron activity, the type of receptors involved and the influence of estradiol milieu on this response in female mice.

Materials and Methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

Animals Female mice expressing GFP under the control of the GnRH promoter were used (187). Mice were on a B6CBA/F1 background and aged from 66 to 126 days. Animals were housed on a 14-h light, 10-h dark cycle with light on at 0400 AM EST. Animals were provided with water and Teklad 2916 chow (Envigo, Madison, Wisconsin,

USA) *ad libitum*. To study effect of estradiol on stress response, mice were ovariectomized (OVX) under isoflurane anesthesia with bupivacaine as a local analgesic. Mice were either implanted with a Silastic (Dow Corning, Midland, MI, USA) capsule containing 0.625 μg of 17β-estradiol in sesame oil (OVX+E group) or not treated further (OVX) at the time of surgery (12). Recordings were performed 2-3 days after surgery. The Institutional Animal Care and Use Committee of the University of Michigan approved all procedures.

Brain slice preparation Solutions were bubbled with 95% O_2 and 5% CO_2 throughout the experiments and for at least 15 min before exposure to tissues. The brain was removed rapidly 1.5 to 2h before lights off and placed in ice-cold sucrose saline solution containing 250 mM sucrose, 3.5 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.25 mM Na_2HPO_4, 1.2 mM $MgSO_4$, and 3.8 mM $MgCl_2$. Coronal brain slices (300 μm) were prepared with a Leica VT1200S (Leica Biosystems, Buffalo Grove, IL, USA). Slices were incubated in a 1:1 mixture solution of sucrose-saline and artificial cerebrospinal fluid (ACSF) containing 135 mM NaCl, 3.5 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.25 mM Na_2HPO_4, 1.2 mM $MgSO_4$, and 2.5 mM $CaCl_2$ for 30 minutes at room temperature and then transferred to 100% ACSF for at least 30 minutes at room temperature before recording. Slices were placed in the chamber continuously perfused with oxygenated ACSF at a rate of 3 ml/min and heated by an in-line heater (Warner Instruments, Hamden, CT, USA) to maintain temperature at 30±1 °C. GFP-labeled GnRH neurons were identified by brief fluorescent illumination at 488 nm on an upright fluorescence microscope Olympus BX51W1 (Opelco, Dulles, VA, USA).

Electrophysiological recordings Recording pipettes were pulled from borosilicate capillary glass (type 7052, 1.65-mm outer diameter and 1.12-mm inner diameter; World Precision Instruments, Inc., Sarasota, FL, USA) using a Flaming/Brown P-97 (Sutter Instrument, Novato, CA, USA) to obtain pipettes with a resistance of 2 to 3.5 MΩ when filled with HEPES-buffered solution, containing 150 mM NaCl, 3.5 mM KCl, 10 mM HEPES, 10 mM glucose, 1.3 mM $MgCl_2$, and 2.5 mM $CaCl_2$. Targeted single-cell extracellular recordings were performed with an EPC-8 or EPC-10 dual-patch clamp amplifier (HEKA Elektronik, Holliston, MA, USA) and Patchmaster software (HEKA Elektronik) as data acquisition software. This method does not alter the intracellular milieu of the cell and minimizes interaction between the recording pipette and cell membrane (188,189). Low resistance seals were made between recording pipette and neuron. Seal resistance was checked every 10 minutes during the recording. Data were excluded if the resistance was >25 MΩ. Recordings were made in voltage-clamp mode at 0 mV holding potential and signal filtered at 10 kHz. Data were obtained from 9 to 12 cells per treatment group, with no more than two cells per mouse; range of firing rate within an animal was similar to that between animals within a group. All recorded neurons were mapped to a brain atlas (190) to determine the relation between anatomical location and response to treatment. No correlation between location of cells and response was observed in this study.

Experimental design GnRH neurons from OVX+E mice show time-of-day-dependent change in firing activity; the firing rate is low in the morning due to estradiol negative

feedback and high in the late afternoon due to estradiol positive feedback (12). To study effects of CRH on GnRH neuron firing activity, recordings were made in late afternoon when mice treated with estradiol exhibit positive feedback (12). After establishing the extracellular recording configuration, recordings were stabilized for 5-10 min and then spontaneous basal (control) activity was recorded for 5 min before treatment. CRH at various doses (0 (i.e., vehicle), 10, 30, 100 or 1000 nM; Bachem, Torrence, CA, USA) was then bath-applied for 5 min, followed by a wash period lasting up to 20 min to determine if effects were reversible. To study the CRH receptor (CRHR) subtypes that contribute to effects of CRH on GnRH neuron firing activity, specific agonists were used. The CRHR-1 agonist stressin I (10 nM; Bachem) or the CRHR-2 agonist urocortin III (10 nM; Bachem) were bath-applied to separate sets of brain slices from OVX+E mice in place of CRH in the above paradigm. If no firing activity was observed during the wash out period, ACSF with 20 mM K^+ was applied to induce firing to verify cell viability and recording integrity. If a cell did not display action currents in response to high K^+ treatment, data from that cell were excluded from the analysis. All drug stocks (CRH, stressin I, urocortin III) were reconstituted in water and diluted at least 1:1000 in ACSF for treatments.

Data analysis Action currents during targeted-extracellular recording reflect action potential firing. Action currents were detected using custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Data were binned at 60-s intervals and mean firing rate (number of action potentials/recording duration) was calculated for control (last 3 min of control period), treatment (last 2 min of treatment and first min of wash out

period; washout period begins when the intake is switched back to ACSF and treatment exposure continues unaltered for at least another minute before the solution change reaches the chamber), and wash out periods. The wash was divided into sequential 3-min periods (skipping the first 2 min to allow solution exchange) to equalize the duration of recording for analysis. The first wash period in which mean frequency returned to baseline levels was used for analysis; the last wash period was used for cells in which firing rate did not completely recover from treatment. Percent change in mean firing frequency during treatment was calculated relative to that during the control period. Cells were defined as responding if firing frequency during treatment changed by ≥ 30%; both responding and non-responding cells were included in statistical analyses for each treatment. In addition to mean firing rate, changes in burst firing characteristics of GnRH neuron were also determined. Groups of action currents (bursts) were identified by custom software in Igor Pro. This software adjusted the maximum time between events (burst window) from 0.01 to 5 s at 10 ms intervals. Events are included in a burst if the time interval between events is less than or equal to the burst window. For the present analyses, action currents were included together as a burst if the interval between events was ≤ 320 ms. This burst window was chosen because it provides the consistent maximum number of bursts detected by the software across the groups in this study. Burst frequency (bursts/3 min), burst duration, number of spikes/burst, and intraburst interval were analyzed and compared between control, treatment, and wash out periods.

Statistics Data are reported as mean±SEM. Statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA, USA). Normality of data was analyzed using a Shapiro-Wilk normality test. Two-way repeated-measure ANOVA was used to determine effects of CRH 30 and 100 nM on firing activity during control, treatment, and wash out periods of GnRH neurons between OVX and OVX+E mice. Student's paired t test was used to analyze the effect of 1 μM CRH on GnRH neurons from OVX mice (no wash period was included for these recordings as the lack of effect was obvious). Effects of CRHR-specific agonists (stressin I and Ucn III) or vehicle on firing activity of GnRH neurons were analyzed by one-way repeated-measure ANOVA for control, treatment and wash out periods. Burst parameters as defined above were analyzed by two-way repeated-measure ANOVA for control, treatment, and wash out periods. Specific statistical tests are indicated in the figure legends. Significance was set at $P<0.05$.

Results

CRH does not affect GnRH neuron firing activity in OVX mice

To study the effect of CRH on GnRH neurons in the absence of ovarian estradiol, we tested effects of low (30 nM) and high (100 nM) concentrations of CRH on GnRH neuron firing activity in brain slices from OVX mice. Figures 2-1A-C show representative traces from extracellular recording of GnRH neurons from OVX mice before and during treatment with CRH. A minority (2 of 9) of GnRH neurons from OVX mice treated with 30 nM CRH exhibited increased firing by the 30% change criteria but overall this treatment had no effect on firing rate (Figure 2-1D, E, n=9 control 0.3±0.1 Hz, CRH 30

nM 0.4±0.1 Hz, wash 0.4±0.1 Hz, P>0.9). Similarly, in response to 100 nM CRH, 2 of 9 GnRH neurons from OVX mice exhibited decreased firing, but overall 100 nM CRH had no effect on firing rate (Figure 2-1D, E, n=9 control 0.4±0.1 Hz, 100 nM CRH 0.4±0.2 Hz, wash 0.5±0.1Hz, P>0.9). To examine if the lack of response in cells from OVX mice was due to insufficient dose, 1000 nM CRH was tested in this group; this dose had no effect on firing frequency of GnRH neurons from OVX mice (Figure 2-1D, E, n=5 control 0.20±0.03 Hz, 1 μM CRH 0.20±0.03 Hz, P>0.8).

CRH alters firing activity of GnRH neurons from OVX+E mice in dose-dependent manner

To study if CRH affected GnRH neuron activity in the presence of estradiol, CRH was bath-applied to brain slices from OVX+E mice; no steroids were added during recordings, estradiol was present only *in vivo*. Representative traces of GnRH neuron firing activity during control, CRH treatment, and wash out periods are shown in Figures 2A and B. In cells from OVX+E mice, 30 nM CRH increased the firing frequency in 4 of 9 cells and increased overall firing rate of the group (Figure 2-2D, E, n=9, control 0.7±0.1 Hz, CRH 30 nM 1.2±0.5 Hz, wash 0.7±0.2 Hz, P<0.05). In marked contrast, 100 nM CRH decreased the firing frequency of 7 of 12 GnRH neurons from OVX+E mice (Figure 2-2D, E, n=12, control 0.8±0.2 Hz, CRH 100 nM 0.4±0.1 Hz, wash 0.8±0.2 Hz, P<0.01). During 100 nM CRH treatment, 2 of 12 cells showed increased in firing frequency, but the overall effect of 100 nM CRH was inhibitory. Because estradiol appeared to induce sensitivity of the GnRH firing response to CRH, we tried a lower CRH dose on slices from OVX+E mice. GnRH neurons did not respond to 10 nM CRH

treatment (n=5, control 0.4±0.2 Hz, CRH 10 nM 0.4±0.2 Hz, P>0.8). Similarly, vehicle treatment had no effect on GnRH neuron firing frequency (Figure 2-2D, E, n=5, control 0.63±0.14 Hz, vehicle 0.56±0.12 Hz, P>0.1). These results suggest that CRH alters GnRH neuron activity in an estradiol-dependent manner with both stimulatory and inhibitory actions observed depending upon dose. Further, the consistent lack of response of cells from OVX mice to CRH and of OVX+E mice to 10 nM CRH or vehicle indicates that the changes attributed to 30 and 100 nM CRH in cells from OVX+E mice are not due to random fluctuations in firing rate of GnRH neurons (12,191).

CRH stimulates GnRH neuron firing activity via CRHR-1

Two types of CRH receptors have been identified in mammals: CRH receptor type-1 and type-2 (CRHR-1 and CRHR-2) (43,192). CRHR-1 has a higher affinity for CRH (193). Thus, it is possible that low concentrations of CRH might activate mainly CRHR-1 and lead to stimulation of GnRH neurons, whereas higher concentration of CRH could also bind and activate CRHR-2 and result in a different response. To test if CRH stimulates firing of GnRH neurons via CRHR-1, stressin I (CRHR-1 specific agonist) was bath-applied to brain slices from OVX+E mice and GnRH neuron firing activity recorded. Figure 2-3A shows representative traces from GnRH neurons in OVX+E before and during stressin I (10 nM) treatment. Stressin I increased firing rate of GnRH neurons in 7 of 11 cells from OVX+E mice and no cells exhibited decreased firing rate (Figure 2-3C, D, n=11, control 0.7±0.2 Hz, stressin I 1.0±0.2 Hz, wash 0.8±0.2 Hz, P<0.05). These data support the postulate that low concentrations of CRH activate GnRH neuron activity via the CRHR-1 pathway.

CRH inhibits GnRH neuron firing activity via CRHR-2

To test if activation of CRHR-2 inhibits GnRH neurons, urocortin III (Ucn III), which has been described as an endogenous ligand specific for CRHR-2 (48), was bath-applied to brain slices from OVX+E mice. Ucn III (10 nM) suppressed firing frequency of GnRH neurons in 7 of 11 cells and no cells exhibited increased firing (Figure 2-3B-D, n=11, control 0.9±0.3 Hz, Ucn III 0.5±0.2 Hz, wash 0.9±0.3 Hz, $P<0.05$). This suggests that inhibitory effects of CRH on GnRH neuron activity are at least in part mediated through CRHR-2.

Activation of CRH receptors alters short-term firing pattern of GnRH neurons

The change in mean firing frequency provides an overview of the effects of CRH on GnRH neuron activity. The short-term pattern of how action currents are grouped together, referred to as burst firing, is also of interest and has been associated with hormone secretion from neuroendocrine cells (194). To further understand the effect of CRH on GnRH neurons, burst firing patterns of the above GnRH neurons from OVX and OVX+E mice were analyzed during control, CRH and CRH receptor agonist treatments, and wash out periods. All cells were analyzed for burst frequency in all three periods. Of 61 cells studied, 59 cells exhibited bursts during all three periods and were included in the analysis of burst properties. One cell that exhibited bursts only during the control period, and one cell that exhibited bursts during control and treatment periods are shown in Figure 4 but not included in the repeated-measures analysis for burst properties as they had no bursts to analyze; these cells both fired single spikes

throughout the recording. Of note, two-group analysis of control vs. treatment and three-group analysis of control, treatment and washout revealed statistical differences in the same parameters. In GnRH neurons from OVX mice, there was no change in any burst parameter analyzed in response to any treatment. In GnRH neurons from OVX+E mice, however, CRH (30 nM) and stressin I increased burst frequency (Figure 2-4A). In contrast, 100 nM CRH and Ucn III decreased burst frequency (Figure 2-4A). There was no change in burst duration, spikes/burst or intraburst interval after 100 nM CRH and UcnIII treatment (Figure 2-4B-D). CRH 30 nM increased both burst duration and spikes/burst (Figure 2-4C and 2-4D). Stressin I also decreased intraburst interval and mean burst duration in GnRH neurons (Figure 2-4B and 2-4C). Although the difference in intraburst interval caused by stressin I treatment achieved a P value indicating statistical significance, the absolute magnitude of the change is small and may have minimal biological effect.

Discussion

GnRH neurons integrate a variety of upstream inputs to produce a secretory output pattern that regulates the downstream reproductive system. Stress exposure can increase or decrease reproductive neuroendocrine output, and response to stress is also modulated by steroid milieu. Here we show that both stimulatory and inhibitory effects of stress can be induced by central interactions among neuroendocrine systems. Specifically, CRH can increase or decrease GnRH neuron firing rate; these effects are estradiol dependent and the differential response is mediated through activation of specific CRH receptors.

CRH had a dose-dependent effect on firing rate of GnRH neurons from estradiol-treated OVX mice, with higher concentrations suppressing activity and lower concentrations stimulating it. The effects of CRH on GnRH neurons are mitigated in OVX mice, suggesting estradiol enables the mechanisms that mediate response to CRH. These findings support previous work demonstrating an effect of estradiol on the stress response and extend those studies to effects at the GnRH neuron. At the whole animal level, the inhibitory effect of stress or CRH treatment on LH pulse frequency is stronger in animals with elevated estradiol levels in several species, whether these are due to estradiol treatment or studies in the follicular vs luteal phase of the cycle (77,81,93,110,111,181,195). The mechanisms engaged by estradiol to facilitate the GnRH neuron response to stress are not known but several possibilities exist. First, estradiol may modulate CRH receptor expression. CRH receptor is higher during proestrus, when estradiol peaks during the cycle, than in diestrus (102). The CRHR-2 promoter contains a classical estrogen receptor response element (104), which could be a target for regulation of expression. Although GnRH neurons lack estrogen receptor alpha (ER-α), both estrogen receptor beta (ER-β) and membrane estrogen receptors have been reported in GnRH neurons (134,196). It is thus possible that estradiol acts directly on GnRH neurons to alter CRH receptor expression in these cells. Second, estradiol alters ionic conductances in GnRH neurons, including voltage-gated potassium and calcium currents, transient receptor potential channels and hyperpolarization-activated channels (197-202). In other neurons, CRH adjusts excitability by modulating multiple currents (203,204). Interactions between CRH actions and estradiol-induced changes in intrinsic properties of GnRH neurons could poise these cells to change firing

activity. Third, estradiol may facilitate GnRH neuron response by altering synaptic connectivity from afferent populations that are CRH-sensitive. Estradiol alters synaptic interactions in several brain regions, including the hypothalamus (205). Of interest in this regard, kisspeptin stimulation of GnRH neuron firing activity is greater in OVX+E than OVX mice because in the former kisspeptin increases excitatory neurotransmission to GnRH neurons, in addition to direct effects on GnRH neurons observed in both animal models (140,206). Fourth, estradiol increases CRH mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus in rats (77). This does not apply to the design of the present study because CRH was applied as a treatment, but estradiol upregulation of CRH levels could enhance the effect of stress on reproductive system *in vivo*.

The effects of CRH on GnRH neurons observed in this study could be direct and/or indirect. CRH-containing fibers synapse on GnRH neurons, which express CRH receptor type-1, enabling direct action (125-127). Fibers from CRH-producing neurons in the PVN appose GnRH neurons (125). The source of CRH that acts on GnRH neurons remains controversial, however, as retrograded tracing with cholera toxin from the preoptic area revealed only sparse overlap with CRH neurons in PVN (130). This very sparse PVN CRH input to GnRH neurons may be due to the relative underinnervation of GnRH neurons (1), because direct innervation of GnRH neurons by CRH arises from a different source, and/or because PVN CRH neurons act on GnRH neurons via intermediate cells. Indirectly, GnRH neurons receive numerous upstream inputs and integrate those signals to regulate the downstream reproductive system. Some of these inputs may be CRH-sensitive. GABA and glutamate fast synaptic

transmission are the main forms of communication in the brain (207,208) and GnRH neurons receive both types of fast synaptic inputs (157,209). Blocking $GABA_A$ receptors in the preoptic area *in vivo* eliminates the ability of CRH to reduce LH pulse frequency (162,163); because the action of GABA on GnRH neurons is excitatory (153) this suggests receptors on other cells exhibiting the more typical inhibitory response to GABA mediate this response. Stress modifies glutamatergic synapses in other brain regions (210). In addition to fast synaptic transmission, stress may affect neuromodulation of GnRH neurons. In this regard, both the stimulatory kisspeptin system (140,142,211), and inhibitory gonadotropin-inhibitory hormone (GnIH) system (166,167,212,213) are affected by stress. Kisspeptin neurons both in the arcuate and anteroventral periventricular nucleus regions are reported to express CRH receptors (150). Stress exposure or CRH treatment decrease *Kiss1* and *Kiss1r* expression in the hypothalamus (65), possibly reducing excitatory neuromodulation of GnRH neurons via kisspeptin. Although only a small percent (13%) of GnIH neurons express CRH-R1 receptors (171), over half of GnIH neurons have increased Fos expression in response to stress exposure (172); stress also increased the number of appositions from GnIH-immunoreactive fibers on GnRH neurons (172). These changes would potentially enhance effects of this inhibitory system. Action of CRH via GnRH neuron afferents might explain why some GnRH neurons in this study did not respond to CRH treatment; specifically in some brain slices, upstream cells that are CRH-responsive might be removed during slice preparation.

The dose dependency of the response to CRH suggested the possibility that different CRH receptors may mediate these responses. We used receptor-specific agonists to

test this and found that activation of CRHR-2 inhibits GnRH neurons, whereas CRHR-1 stimulates GnRH neurons. The involvement of two receptors mediating opposite effects may explain some of the variation observed when CRH itself was used as a treatment. The primary effect of 100 nM CRH was inhibitory, but a couple of cells increased firing in response to this treatment; in contrast, none of GnRH neurons treated with the CRHR-2 specific agonist Ucn III, exhibited stimulation. CRH may activate both receptor subtypes and, in most cases, the inhibitory effects of CRH overpowered the stimulatory effect resulting in suppression of GnRH neuron activity. Similarly, when the specific CRHR-1 agonist was used, a higher percent of GnRH neurons responded than did to 30 nM CRH and all responding cells increased firing rate.

The receptor-specific effects of CRH on GnRH neuron firing may be attributable to signaling pathways engaged upon CRH agonist binding. CRH receptors are class-B G-protein coupled receptors that can interact with several Gα subunits (214,215). CRHR-1 appears to primarily couple to Gαs and Gαq (216,217). CRHR-2, however, couples with multiple types of G-proteins, including Gαq (217,218), Gαs (219), and Gαi (217). The type of Gα subunit coupled to CRHR-2, and to some extent CRHR-1, varies among cell types (216). The simplest explanation for the opposite effects of activating CRHR-1 vs CRHR-2 would be direct action on GnRH neurons via Gαs and Gαi subunits. Beyond the current lack of evidence for CRHR-2 expression in GnRH neurons, this explanation does not take into account possible indirect action via afferents that excite or inhibit GnRH neurons. For example, activation of GnRH neuron firing by CRHR-1 agonists may be due to stimulation (via Gαs or Gαq) of an excitatory input. Suppression of GnRH neurons by CRHR-2 agonists may be due to suppression (via Gαi) of an

excitatory input or activation (via Gαs and Gαq) of an inhibitory input. Future work to determine the cell signaling pathways in GnRH neurons and their upstream regulators upon activation of CRHRs are needed to test these postulates.

The ability of CRH to both activate and inhibit GnRH neurons could contribute to the different response of the reproductive system to stress. The importance of CRHR-2 in inhibitory effects of stress on fertility has been noted in several studies (85,86,116). Pretreatment with a CRHR-2 antagonist prevents stress-induced suppression of LH pulses by metabolic (insulin-induced hypoglycemia), immunological (lipopolysaccharide), and psychological (restraint) stressors (85). Urocortin II (Ucn II), which exhibits high affinity for CRHR-2 and low affinity for CRHR-1 similar to Ucn III (48,220), suppresses LH pulses in female rats in a dose-dependent manner and blocking of CRHR-2 abolishes the effect of Ucn II on LH pulses (86). Interestingly, expression of mRNA encoding Ucn II in the PVN is increased following restraint stress (221). A role for CRHR-1 in stimulatory effect of stress on reproductive system *in vivo* has also been reported. Exposure to restraint stress on the morning of proestrus induces LH and FSH secretion in female proestrous rats (90). This stimulatory effect of restraint stress on LH secretion is blocked by pretreatment of CRHR-1, but not CRHR-2, antagonist suggesting CRHR-1 mediates the stimulatory effect of stress. The present results support and extend these previous findings by demonstrating that activation of CRHR-1 vs CRHR-2 can induce or suppress firing of GnRH neurons, respectively.

Burst firing in neuroendocrine cells is related to the secretion of hormones (194,222,223). GnRH neurons exhibit burst firing pattern, although the burst firing in GnRH neurons is slower (224,225) compared to other neurons (226-228). Treatments

that inhibited mean firing rate of GnRH neurons (100 nM CRH or Ucn III) did so by reducing the number of bursts; once initiated, burst duration, number of spikes per burst, and intraburst interval were not different. This may suggest CRHR-2 agonists act indirectly to reduce excitatory drive to GnRH neurons. This would decrease initiation of action potentials and hence number of bursts, but once the burst is initiated, burst characteristics would remain similar because intrinsic properties of GnRH neurons are unaffected. In contrast to CRHR-2, activation of CRHR-1 by 30 nM CRH or stressin I increased burst firing in GnRH neurons. Further, these treatments altered other burst parameters. This suggests a possible direct action via CRHR-1 on GnRH neurons to change intrinsic properties and burst characteristics. Consistent with these postulates and as mentioned above, CRH receptor type 1 mRNA, but not type 2, appears to be expressed in GnRH neurons from female mice (127). Although CRH did not alter mean firing frequency of GnRH neurons from OVX mice, it is possible to alter burst firing without affecting the overall frequency, but we did not observe any change in burst parameters in GnRH neurons from OVX mice during either 30 nM or 100 nM CRH treatments. This emphasizes the importance of estradiol in the response of GnRH neurons to CRH.

The relationship between stress and reproduction varies among studies. The present findings that CRH exerts either stimulatory or inhibitory effects on GnRH neuron activity depending on the CRH receptor activated provide mechanistic insight into how stress can have different effects. The finding that estradiol potentiates both effects of CRH on GnRH neurons supports and extends previous work indicating steroid milieu alters the response to stress. Together, these observations indicate interactions among the stress

and reproductive axes are bidirectional. These findings can help shape future studies of the mechanisms that underlying the difference responses resulting from stress exposure in animals

Figures and Legends

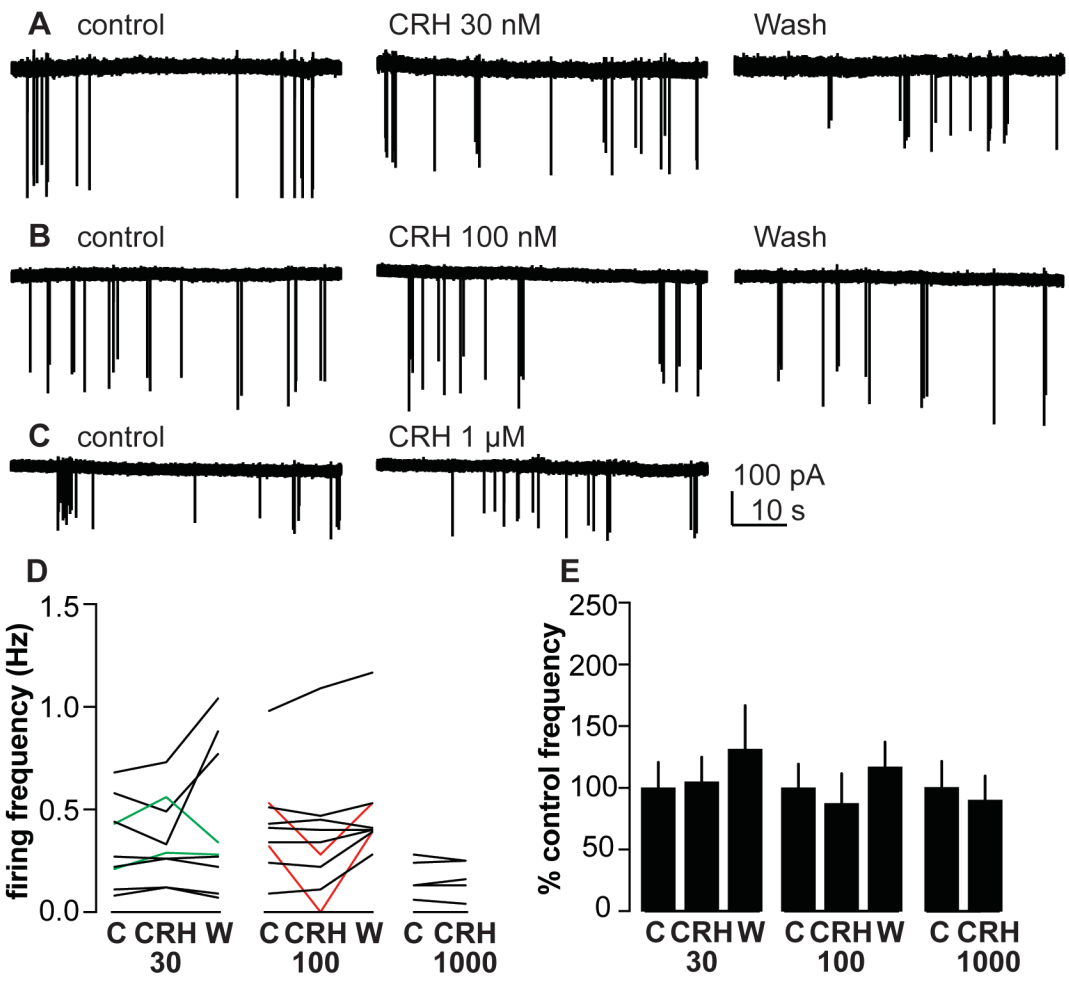

Figure 2-1. CRH has no effect on the firing rate of GnRH neurons from OVX mice. A-C, representative raw data from extracellular recordings of GnRH neurons during each period for 30 nM (A) 100 nM (B) and 1000 nM (C). D, firing rate of individual GnRH neurons during control (C), CRH treatment (CRH30, CRH100, or CRH1000), and wash out (W) periods. Green and red lines indicate cells with ≥30% increase or decrease in firing frequency during treatment, respectively. E, Mean±SEM percentage of control firing frequency. Green and red lines indicate cells with ≥30% increase or decrease in firing frequency during treatment, respectively. No differences were detected for either firing frequency ($P>0.5$, two-way repeated-measures ANOVA/Tukey (30, 100 nM) or paired Student's t test (1000nM), or % of control frequency ($P>0.1$, Friedman test/Dunn's post-hoc or paired Student's t test).

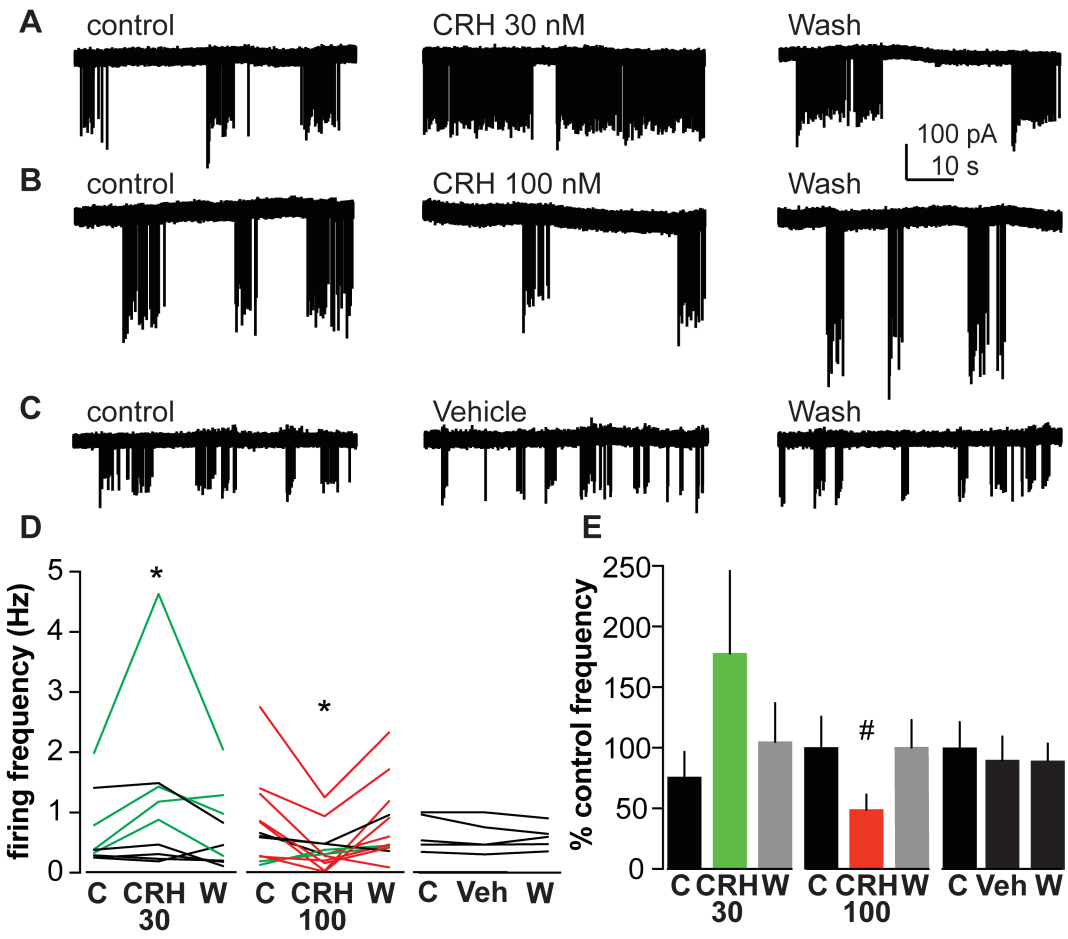

Figure 2-2. CRH has dose-dependent effects on firing activity of GnRH neurons from OVX+E mice. A-C, representative raw data from extracellular recordings of GnRH neurons during each period. D, firing rate of individual GnRH neurons during control (C), CRH treatment (CRH30 or CRH100) or vehicle treatment (Veh), and wash out (W) periods. Green and red lines indicate cells with ≥30% increase or decrease in firing frequency during treatment, respectively. *, P<0.05 mean firing frequency compared to control period, two-way repeated-measures ANOVA/Tukey. E, Mean±SEM percentage of control firing frequency. #, P<0.05 compared to control period, Friedman test/Dunn's post-hoc.

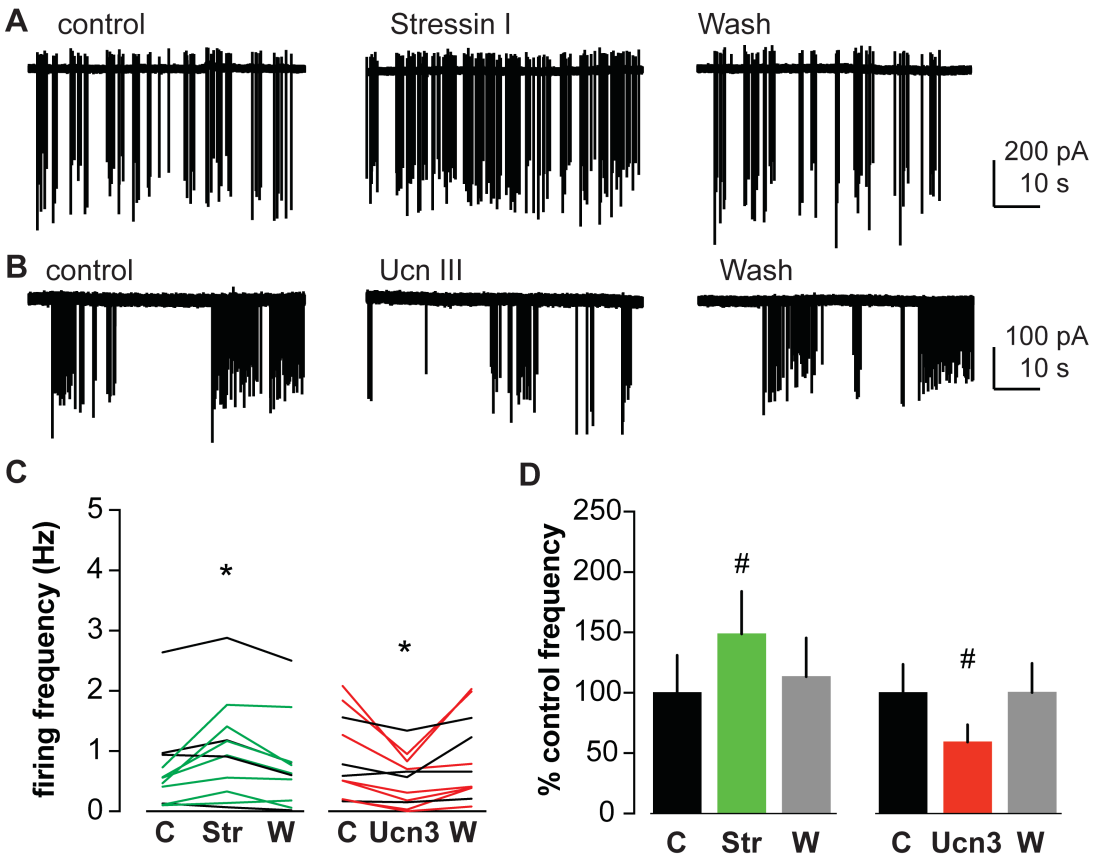

Figure 2-3. Activation of specific CRH receptors has different effects on GnRH neuron firing activity. A and B, representative raw data from extracellular recordings of GnRH neurons during control (left) and CRH receptor agonist treatment periods (stressin 1, top right; ucn III bottom right). C, firing frequency of individual GnRH neurons during control (C), treatment (Str: stressin I or Ucn3: Ucn III), and wash out (W) periods. Green and red lines indicate cells with 30% increase or decrease in firing frequency during treatment, respectively. *, P<0.05 mean firing frequency compared with control period, two-way repeated-measure ANOVA/Tukey. D, Mean±SEM percentage of control firing frequency. #, P<0.05 compared with control period, Friedman test/Dunn's.

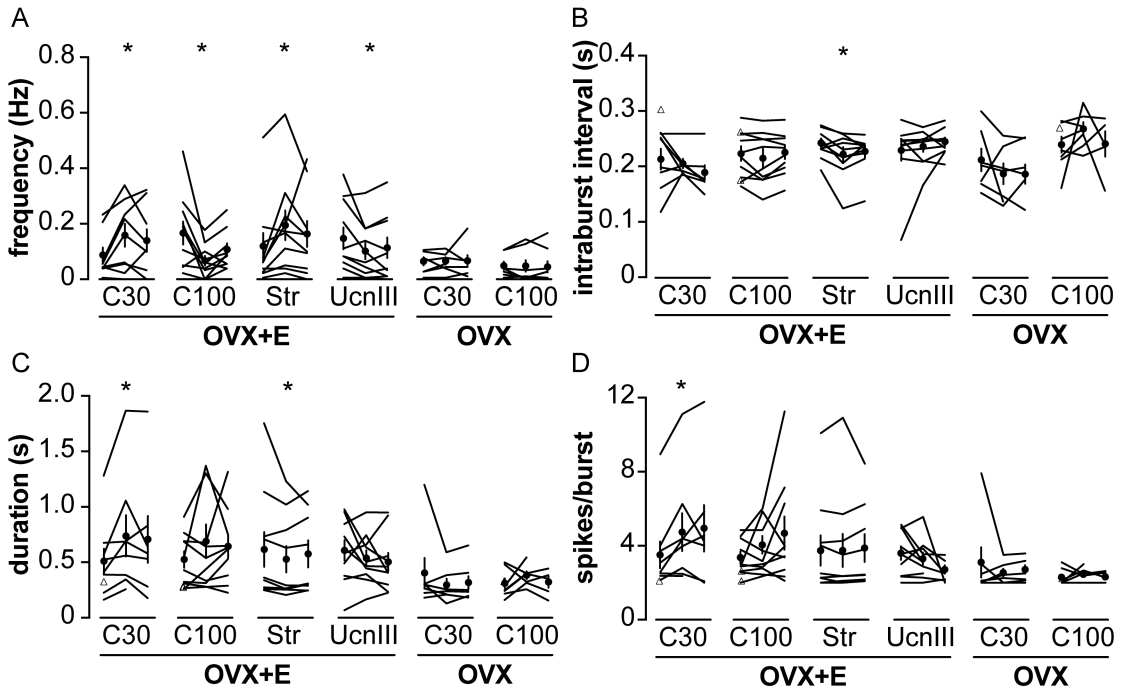

Figure 2-4. CRH alters GnRH neuron burst firing pattern in OVX+E mice. A. Burst frequency of individual GnRH neurons during the control, treatment and wash periods analyzed in Figures 1-3. B-D Intraburst interval (B), burst duration (C) and spikes/burst (D) of individual GnRH neurons. Values from the same cell are connected by lines. Mean ± SEM are shown as a circle with error bar in A-D. Triangles in B-D indicate control burst parameter values of a cell in the C30 group that had bursts during the control period but not during CRH treatment (grey line in A). Dashed lines in A-D connect control and treatment burst parameters of a cell in the C30 group that exhibited bursts during the control and treatment periods but only single spikes during the wash period. C30, 30 nM CRH; C100, 100 nM CRH; Str, stressin I; UcnIII, urocortin III; *, $P<0.05$ control vs treatment, two-way repeated-measure ANOVA/Tukey for CRH treatments and Friedman test/Dunn's for stressin I and UcnIII.

Chapter 3: Neurobiological Mechanisms Underlying the Effects of Corticotropin-releasing Hormone (CRH) on Gonadotropin-releasing Hormone (GnRH) Neuron Function

Abstract

GnRH neurons are central regulators of reproduction and respond to factors affecting fertility, such as stress. Corticotropin-releasing hormone (CRH) is released during stress response. In brain slices from unstressed controls, CRH has opposite, estradiol-dependent, effects on GnRH neuron firing depending on the CRH receptor activated; activating CRHR-1 stimulates whereas activating CRHR-2 suppresses activity. We investigated possible direct and indirect mechanisms. Mice were ovariectomized and either not treated further (OVX) or given a capsule producing high positive feedback (OVX+E) or low negative feedback (OVX+low E) physiologic circulating estradiol levels. We tested possible direct effects on GnRH neurons by altering voltage-gated potassium currents. Two types of voltage-gated potassium currents (transient I_A and sustained I_K) were measured; neither CRHR-1 nor CRHR-2 agonists altered potassium current density in GnRH neurons from OVX+E mice. Further, neither CRH nor receptor-specific agonists altered action potential generation in response to current injection in GnRH neurons from OVX+E mice. To test the possible indirect actions, GABAergic postsynaptic currents were monitored. A CRHR-1 agonist increased GABAergic transmission frequency to GnRH neurons from OVX+E, but not OVX, mice, whereas, a

CRHR-2 agonist had no effect. Finally, we tested if CRH alters the firing rate of arcuate kisspeptin neurons, which provide an important excitatory neuromodulatory input to GnRH neurons. CRH did not acutely alter firing activity of these neurons from OVX, OVX+E or OVX+low E mice. These results suggest CRH increases GnRH neuron activity in an estradiol-dependent manner in part by activating GABAergic afferents. Mechanisms underlying inhibitory effects of CRH remain unknown.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons are the central output components of the hypothalamo-pituitary-gonadal (HPG) axis. GnRH is released in a pulsatile manner that stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone, which control steroidogenesis and gametogenesis. GnRH neurons integrate several inputs that regulate their activity, hormone release and thus reproductive function. One such signal is stress. Most research indicates stress inhibits reproductive parameters, such as LH and/or GnRH pulses, multiunit activity (MUA) associated with LH release, reproductive cycles, and the preovulatory LH surge that triggers ovulation (70,93,229-235). There are several examples, however, where stress can activate aspects of the reproductive system in an acute manner (114,115,177,236,237). Further, sex steroid milieu sculpts the response of the reproductive system to stress, suggesting bidirectional interactions of these systems (77,81,111). Circulating estradiol, in particular, appears to potentiate the effects of stress (77,81,111).

Stress activates several pathways, including the hypothalamic-pituitary-adrenal (HPA) axis, triggering release of corticotropin-releasing hormone (CRH, also known as corticotropin-releasing factor, CRF). CRH may mediate inhibitory effects of stress on reproduction. Pretreatment with individual or combined CRH receptor antagonists blocks effects of different stressors on LH pulses (85,86), and intracerebroventricular injection of CRH suppresses LH pulses (a bio-readout of GnRH pulses) (237). In some instances, however, CRH can acutely activate the reproductive system. For example, central injection of CRH increases LH pulse frequency in both male and female gonadectomized sheep supplemented with estradiol (88). One third of ovariectomized monkeys injected with CRH exhibited premature, short-duration hypothalamic multiunit activity volley (237), indicating central stimulatory effects of CRH.

To examine how stress interacts mechanistically with the neuroendocrine control of reproduction, we focused on its effects on estradiol positive feedback induction of the LH surge. Exposure to a layered, psychosocial stress paradigm on the morning of proestrus disrupted the LH surge in about two-thirds of mice (119). Interestingly, uterine mass in these mice was indistinguishable from animals that exhibited LH surges. Because uterine mass is linearly correlated with estradiol levels (120), this suggested a failure to process rather than a failure to generate the estradiol rise. Consistent with this, the same layered stress paradigm disrupted the estradiol-induced LH surge using an established daily LH surge model (119). Using this same daily surge model in unstressed mice, CRH was shown to stimulate or inhibit the firing activity of GnRH

neurons depending on the type of CRH receptor (CRHR) activated; these effects were dependent upon circulating estradiol (238).

Here we extended these studies to examine the neurobiological mechanisms underlying the stimulatory and inhibitory effects of activation of CRHR on GnRH neuron firing rate. We examined potential direct effects via voltage-gated potassium currents in GnRH neurons and the excitability of these cells, and potential indirect mechanisms via GABAergic and arcuate kisspeptin-neurokinin B-dynorphin (KNDy) afferents. We further examined if circulating estradiol modulates the effects of CRH receptor activation on the afferent populations studied.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Animals The Institutional Animal Care and Use Committees of the University of Michigan or the University at Albany approved all procedures. Mice aged from 62 to 120 d were used for all experiments. No correlation between the age of animals and the response to treatment was observed in any study. Adult transgenic female mice expressing green fluorescent protein (GFP) under the control of GnRH promoter (GnRH-GFP, JAX 033639) or eGFP under control of the *Tac2* promoter (Tac2-GFP, 015495-UCD/STOCK Tg [*Tac2*-EGFP]381 Gsat) were used for electrophysiology. *Tac2* encodes tachykinin 2, which is a precursor for neurokinin-B. Single-cell PCR

demonstrates that fluorescently-identified cells in the relatively thick brain slices used for recording in the Tac-2-GFP mouse model also express kisspeptin and/or dynorphin at high percentages in support of their identity as KNDy neurons (239). These mice were housed in the University of Michigan vivarium on a 14 h light and 10 h dark cycle with lights on at 04:00 AM Eastern Standard Time and were provided with water and Teklad 2916 chow (Envigo, Madison, WI) *ad libitum*. A transgenic CRHR-1 reporter line in which a bacterial artificial chromosome containing the CRHR-1 promoter was used to drive GFP expression was also used (CRHR-1-GFP; (240)). These mice were maintained in the University at Albany vivarium under 12 h light and 12 h dark cycle with lights on at 07:00 AM Eastern Standard Time, with and were provided with Prolab Isopro RMH 3000 (5P76) chow (Purina, St. Louis, MO) and water available *ad libitum*.

To study the influence of circulating estradiol on responses to CRH and specific receptor agonists, two different steroid feedback models were used. First, an established daily surge model that produces diurnal shifts between estradiol negative feedback in the AM and positive feedback in the PM was used, with mice studied in the PM during positive feedback (119,139,157,199,238,239,241-244). For this, mice were ovariectomized (OVX) under isoflurane anesthesia with bupivacaine as a local analgesic 2-3 d before the day of experiment. At the time of surgery, mice were either implanted with a Silastic capsule (0.078" I.D. x 0.125" O.D., Dow Corning, Midland, MI) containing 0.625 μg of estradiol in sesame oil (OVX+E) or not treated further (OVX) (12,245). In this daily surge model, uterine mass is higher in OVX+E than OVX mice, indicating a difference in functional circulating estradiol levels (GnRH-GFP OVX

45.9±2.8 mg, OVX+E 175.2±2.0 mg, p<0.0001 two-tailed Mann-Whitney U test, U=0; Tac2-GFP OVX 41.9±1.6 mg, OVX+E 136.8±4.7 mg, p<0.0001 two-tailed Mann-Whitney U test, U=0;). Mice used for assessment of CRHR-1 co-labeling with GnRH were implanted with the 0.625 µg estradiol capsules. Second, for some studies (explained below) we adopted a new model that produces negative feedback with exposure to a lower level of estradiol (low E) (117). Mice were OVX+low E using the same procedures, but the implant contained 107 ng estradiol and mice were studied 10-12 days after surgery. Uterine mass from the groups prepared in in this model indicate level of estradiol replacement consistent with diestrus, and a further reduction in uterine mass in OVX mice attributable to longer duration post OVX compared to the daily surge model above (n=5 each, OVX 19.8±0.7 mg, OVX+low E 39.2±0.8 mg, p=0.0079 two-tailed Mann-Whitney U test, U=0).

Experimental design overview The daily LH surge model of estradiol positive feedback (119,139,157,199,238,239,241-244) was used for most studies to examine the neurobiological mechanisms induced by activation of CRH receptors. This model exhibits similar neurophysiologic measures to those that occur in GnRH neurons on proestrus (12,243,246). Because effects of CRH and receptor-specific agonists were dependent on circulating estradiol in past work, studies of GnRH neurons were done in OVX+E mice, with OVX mice examined to test for estradiol dependence if the parameter being monitored was altered in response to acute drug treatment. Both OVX and OVX+E mice prepared in the daily surge model were included in studies of Tac2-GFP neurons to test for estradiol dependence of acute actions of CRH. The low E

model was used to test if the lack of suppressive effect of CRH on Tac2-GFP neurons was because the strong suppressive effect of the daily surge estradiol dose precluded observation of an acute inhibition of activity by treatment. The low E model mimics diestrous uterine mass, diurnal corticosterone patterns and reduces LH pulse frequency (117).

For electrophysiology studies, we focused on females as the effects of stress on fertility are more pronounced; male mice were also included for the immunofluorescence study. The desired recording configuration was achieved, recordings stabilized as specified for each approach, then control measures obtained (vehicle for all treatments was ACSF). Treatment was bath-applied for 5 min. Analysis of treatment effects began 3 min after treatment was initiated (1 min to reach bath and 2 min for solution exchange). This was the time required to observe differences in firing rate in response to CRH or CRHR agonists (238). Treatment was washed out and reversibility assessed 5-15 min after wash began. To probe the mechanisms underlying activation of CRHR-1 (aka CRF_1R) vs CRHR-2 (aka CRF_2R), receptor-specific agonists were chosen. This allowed parallel studies with drugs at an effective dose (10 nM) that exhibited at least 100 fold selectivity for that receptor (48,247). The concentration of was selected based on the literature and was effective in previous studies demonstrating changes in firing activity of GnRH neurons (238) and reconfirmed here to test preparation effectiveness. The CRHR-1 agonist stressin I (10 nM, Tocris) increased GnRH neuron firing (n=6, con 0.34±0.07 Hz, stressin I 0.63±0.17 Hz, P=0.03 two-tailed, Wilcoxon signed rank test, W=21); the CRHR-2 agonist urocortin 3 (10 nM, Bachem, Torrance, CA) decreased GnRH neuron

firing (n=6, con 0.54±0.31 Hz, urocortin 3 0.07±0.02 Hz, P=0.03, two-tailed Wilcoxon signed rank test, W=-21).

Brain slice preparation All solutions were bubbled with 95% O_2 and 5% CO_2 for at least 15 min before exposure to tissue and throughout the experiments. Brains were rapidly removed 11.5-12 h after lights on and placed in an ice-cold sucrose saline solution containing (in mM): 250 sucrose, 3.5 KCl, 25 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal slides (300 μm) were made with a Leica VT1200S (Leica Biosystems, Buffalo Grove, IL). Slices were incubated in a 1:1 mixture of sucrose-saline and artificial cerebrospinal fluid (ACSF) containing (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ for 30 min at room temperature. Slices were then transferred to 100% ACSF at room temperature for at least 30 min before recording. Slices were used within 6 h of preparation. No more than two cells were used per animal and n is defined as number of cells.

Electrophysiology recordings Brain slices were transferred to a recording chamber and perfused with oxygenated ACSF at a rate of 3 mL/min and heated by an in-line heater (Warner Instruments, Hamden, CT) to maintain temperature at 30±1°C. GFP-positive neurons were identified by brief illumination with 470 nm light using an upright fluorescence microscope Olympus BX51W1 (Opelco, Dulles, VA). Recording pipettes were pulled from borosilicate glass pipettes (type 7052, 1.65 mm outer diameter and 1.12 mm inner diameter; World Precision Instruments, Inc., Sarasota, FL) using a P-97 puller (Sutter Instruments, Novato, CA) to obtain pipettes with a resistance of 2 to 3.5

MΩ. All recordings were performed with an EPC-10 dual-patch clamp amplifier and Patchmaster acquisition software (HEKA Elektronik, Holliston, MA). The membrane potential for recordings with 20 or 140 mM chloride pipette solutions were corrected on-line for liquid junction potentials of -14.7 mV and -4.9 mV, respectively. All recorded cells were mapped to a brain atlas (248) to determine whether cell location is related to response to treatment. No correlation between location and response was observed in this study. Whole-cell recording quality was monitored by assessing input resistance (Rin), series resistance (Rs), capacitance (Cm) and holding current (Ihold) every 3 min from the average of 20 traces recorded in response to a 20 ms 5 mV hyperpolarizing step. Only recordings with stable (<20 % change) Rin (>500 MΩ), series resistance (<20 MΩ), Cm (10-35 pF) and Ihold (-60 to 5 pA) were analyzed.

Voltage-gated potassium currents To isolate voltage-gated potassium currents, brain slices were perfused with ACSF supplemented with 2 µM tetrodotoxin (TTX; Tocris), 200 µM $CdCl_2$, 20 µM D-APV (Tocris), 10 µM CNQX, and 100 µM picrotoxin to block fast sodium and calcium currents, and ionotropic GABA and glutamate receptors. GnRH neurons from OVX+E mice were recorded in whole-cell voltage-clamp mode and held at -60 mV between voltage protocols. The current density of two types of voltage-gated potassium currents, the rapid (I_A) and the sustained (I_K), were recorded using abbreviated protocols to minimize drift between control and treatment periods. To record total potassium current, membrane potential was hyperpolarized (500 ms at -110 mV) to remove inactivation of the rapid component followed by a 500 ms prepulse of -110 mV and then the current measured at the test step (250 ms at -10 mV). To record

the sustained current, a depolarized prepulse (500 ms at -40 mV) was applied to inactivate the transient current leaving only sustained current to be recorded during the 50 ms test pulse at -10 mV. All traces were on-line leak subtracted using a P/-4 protocol. To test effects of activating CRH receptor subtypes on voltage-gated potassium currents, I_A and I_K were compared between control and treatment periods. After a 2-min stabilization period, the voltage-gated potassium current protocol was performed five times at 30-s intervals under control conditions, then 10 nM of either stressin I (CRHR-1 agonist; Tocris) or urocortin 3 (CRHR-2 agonist; Bachem, Torrance, CA) starting 3 min after treatment initiation.

Excitability in GnRH neurons Whole-cell current-clamp recordings were performed on GnRH neurons from OVX+E mice in the presence of 20 μM D-APV, 10 μM CNQX, and 100 μM picrotoxin to block ionotropic GABA and glutamate receptors. After achieving stable whole-cell recordings and adjusting bridge balance (95%), direct current (<25 pA, 10.1±1.2 pA) was adjusted to keep the membrane potentials within 2 mV of -70 mV. Membrane response of GnRH neurons was then recorded in response to current injections (0 to 40 pA, 2 pA intervals, 500 ms). This protocol was run three times separated by 1 min during the control period and then treatment (100 nM CRH, 10 nM stressin I, or 10 nM urocortin 3) was bath-applied for 5 min and the protocol run again three times starting 3 min after treatment initiation, then treatment was washed out for 5-10 min and the protocol repeated three more times. The number of action potentials at each current step was averaged and compared among control, treatment, and wash out periods. The first spike fired was used to determine action potential properties:

minimum current needed to induce firing (rheobase), latency from start of the current injection to first spike, firing threshold (defined as 1 V/s), rate of rise, peak amplitude relative to threshold, full-width at half-maximum (fwhm), and time, minimum, and amplitude of the afterhyperpolarization potential (AHP) relative to action potential initiation.

GABAergic postsynaptic currents (PSCs) Whole-cell voltage-clamp recordings were made of GABA PSCs in GnRH neurons held at -65 mV in brain slices from OVX+E and OVX mice. High-resistance seals (≥1.5 GΩ) were made between the pipette tip and cell membrane. Pipettes were filled with a solution containing (in mM): 140 KCl, 10 HEPES, 5 EGTA, 0.1 $CaCl_2$, 4 MgATP, and 0.4 NaGTP, and recordings were done in the presence of 10 μM CNQX and 20 μM D-APV to block ionotropic glutamatergic receptors. After establishing the whole-cell recording configuration, recordings were stabilized for 3 min and then were recorded for a 6-min control period (2x3 min). The CRHR-1 agonist stressin I (10 nM) or the CRHR-2 agonist urocortin 3 (10 nM) were bath-applied for 5 min to brain slices. Data were continuously recorded, but analysis of treatment effects began 3 min after treatment initiation. This was followed by a wash period lasting up to 15 min to determine if any effects were reversible.

Extracellular recordings Targeted extracellular recordings were performed to determine the effect of CRH on firing rate of KNDy neurons. Recording pipettes were filled with HEPES-buffered solution, containing (in mM): 150 NaCl, 3.5 KCl, 10 HEPES, 10 D-glucose, 1.3 $MgCl_2$, and 2.5 $CaCl_2$. Low resistance seals (<25 MΩ) were made between

the recording pipette and neuron. After establishing the extracellular recording configuration, recordings were stabilized for 5-10 min. Basal activity of KNDy neurons from OVX and OVX+E mice were recorded for a 5-min control period, followed by bath-application of 100 nM CRH for 5 min. Data were continuously recorded, but analysis of treatment effects began 3 min after treatment initiation. This was followed by a wash period of up to 15 min. If no firing activity was observed during the wash period, ACSF with 20 mM K^+ was applied to induce firing to verify cell viability and recording integrity. If a cell did not display action currents in response to high K^+ treatment, data from that cell were excluded from the analysis. Recordings of the same duration were also conducted with vehicle to examine stability of firing rate over time since initiation of the recording.

Data analysis Events (PSCs or action currents) were detected using custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Data were binned at 60-s intervals and mean firing rate (number of action currents/recording duration) or mean PSC frequency (number of PSCs/recording duration) were determined for control (last 3 min of control period), treatment (last 2 min of treatment and first min of wash out period when treatment is still present in the slice chamber), and wash out periods (last 3 min of wash out period). Percent changes in mean firing frequency or mean PSC frequency were calculated relative to that during the control period. Cells were defined as responding if the percent change is ≥30%, but both responding and non-responding cells were included in statistical analyses for each treatment. In addition to frequency, PSCs amplitude, decay time, and full width at half-maximum (fwhm) were analyzed. For

voltage-gated potassium currents, peaks of I_A and I_K were determined by customized software written in Igor Pro. I_A was isolated by subtracting the sustained current recorded after the -40 mV prepulse from the total current. Current density was calculated by dividing peak current values by capacitance.

GnRH/CRHR-1-GFP Tissue Processing, Immunofluorescence (IF), Microscopy, and Quantification Dual-label fluorescent IHC was performed to determine if GnRH neurons co-express CRHR-1. Brains were collected from male, diestrous female, and OVX+E 2 days post surgery CRHR-1-GFP mice (n=2/group) and stored in 4% paraformaldehyde. Twenty-four hours later, brains were moved to a 30% sucrose solution in which they were stored until cryostat sectioning at 30 µm into four series. For IF, brain sections were rinsed in phosphate-buffered saline (PBS; pH 7.6), and then placed in a blocking solution consisting of 4% normal donkey serum (4% NDS, Equitech-Bio, Kerryville, TX, USA) with 0.3% Triton-X in PBS (PBS-TX) for 1 h. Sections were then moved to primary antisera for GnRH (EL14; anti-rabbit; kindly provided by Oline Rønnekleiv; RRID: AB_2715535 (249); 1:5000 in block solution) and incubated overnight at room temperature. The following day, sections were rinsed in PBS and incubated in secondary antisera (Jackson ImmunoResearch, West Grove PA); donkey anti-rabbit Alexa 594; 1:500) for 2.5 h. Sections were then placed into the second primary antisera against GFP (Abcam; chicken; RRID: AB300798; 1:2000) at room temperature overnight. The next day, tissue was rinsed in PBS, and placed in secondary antisera (Jackson ImmunoResearch; donkey anti-chicken Alexa 488; 1:1500) for 2.5 h. After rinsing, sections were mounted and coverslipped with Vectashield HardSet mounting

media (Vector Labs, Burlingame, CA). Imaging of GnRH and CRHR-1-GFP was performed on a Nikon 80i microscope at 20X magnification. Images were collected from 12 sections ranging from plate image 44 to 61 from the Allen Institute mouse brain coronal reference atlas (https://mouse.brain-map.org/static/atlas). The distance between sections that were quantified was 240 μm. Total numbers of GnRH neurons and GnRH/CRHR-1-GFP co-labeled neurons were counted bilaterally in these sections.

Statistics Data are reported as mean±SEM. Statistical analyses were performed using Prism 8 (GraphPad Software, La Jolla, CA, USA). Distribution of data was analyzed using a Shapiro-Wilk test. Details on specific tests are provided in the results. Significance was set at $P<0.05$.

Results

Activating CRH receptors does not affect the amplitude of voltage-gated potassium currents in GnRH neurons from OVX+E mice prepared in the daily surge model

Firing rate of GnRH neurons from OVX+E mice is increased by activation of CRHR-1 and decreased by activation of CRHR-2 (238). To test if CRH receptor activation modifies voltage-gated potassium currents in GnRH neurons to bring about these responses, the density of fast transient and sustained potassium currents in GnRH neurons from OVX+E mice was monitored before and during treatment of CRHR-specific agonists. Representative traces are shown in Figures 3-1A and 3-1B. Neither the CRHR-1 agonist stressin 1 (10 nM) nor the CRHR-2 agonist urocortin 3 (10 nM)

altered either I_A or I_K current density in GnRH neurons from OVX+E mice (Figure 3-1C-D, Table 3-1, both n=7, two-tailed, paired Student's *t* test, P>0.1). Passive properties of GnRH neurons were not affected by either stressin I or urocortin 3 (Table 3-1).

Neither CRH nor receptor-specific agonists alter excitability of GnRH neurons from OVX+E mice prepared in the daily surge model

To investigate if CRH more broadly alters the excitability of GnRH neurons, we measured the response of GnRH neurons from OVX+E mice to current injection steps in whole-cell current-clamp recordings. Figure 3-2A shows representative responses to 16 and 24 pA injections. The number of spikes generated in response to current injections from GnRH neurons were not altered by CRH (100 nM, n=7) treatment (Figure 3-2B, Table 3-2). Other action potential parameters were also not affected by CRH treatment (Figure 3-2C-J, Table 3-2). We further tested effects of specific CRHR agonists. Selective activation of CRHR-1 (10 nM stressin I, n=7) did not affect excitability of GnRH neurons or action potential properties (Figure 3-3, Table 3-2). Likewise, the excitability of GnRH neurons was not affected by selective activation of CRHR-2 (10 nM urocortin 3, n=8, Figure 3-4). Of note, 3 of 8 of GnRH neurons (green lines Figure 3-4C) showed an apparent increase in excitability during urocortin 3 treatment. Other parameters (AP width, amplitude, threshold, rate of rise, AHP amplitude, AHP time, and AHP minimum) were not affected by urocortin 3 treatment (Figure 3-4D-J, Table 3-2). Neither CRH nor CRHR agonists affected passive properties of GnRH neurons (Table 3-3). These results indicate that activation of CRH receptors

has no acute effect on the ability of GnRH neurons to generate action potentials in response to current injection.

No colocalization observed between CRHR-1 and GnRH in either females or males

Of a total of 156 GnRH neurons counted from two diestrous females (Figure 3-5A-D) and 117 GnRH neurons counted from two males (Figure 3-5E-H), and 153 counted from two OVX+E females (Figure 3-5I-L), none were found that were co-labeled with CRHR-1-GFP. CRHR-1-GFP neurons were, however, often found in proximity to GnRH neurons. Mice for the immunofluorescent work were housed under a 12:12 light:dark cycle vs 14:10 for electrophysiology, and these mice were gonad intact. It is possible either of these changes could alter receptor expression, however the lack of detectable CRHR-1 within GnRH neurons in both males and females is consistent with the above physiologic studies indicating no effects of stressin I on GnRH neuron intrinsic properties that were examined.

CRH does not acutely alter firing rate of Tac2-GFP-identified cells prepared in the daily surge model

Because arcuate kisspeptin neurons are important upstream regulators of GnRH neurons that have been implicated in the stress response, we next tested if CRH affects the firing rate of Tac2-GFP neurons in this region in brain slices from OVX and OVX+E mice. Figures 3-6A and 3-6B show representative extracellular recording traces of Tac2-GFP neurons from OVX and OVX+E mice before, during and after 100 nM CRH

treatment. As reported (242), basal firing rate of Tac2-GFP neurons was higher in cells from OVX mice than those from OVX+E mice (Figure 3-6C, OVX 0.80±0.19 Hz, n=15 vs OVX+E 0.14±0.05 Hz, n=12, U= 19.5, P<0.001 two-tailed, Mann-Whitney U test). In OVX+E mice, CRH did not affect Tac2-GFP neuron firing rate (Figure 3-6D, Table 3-4 for ANOVA parameters, control 0.10±0.05 Hz, CRH 0.10±0.04 Hz, wash 0.15±0.06 Hz, n=7, P>0.9, two-way repeated-measures ANOVA/Tukey, all comparisons). In OVX mice, the firing rate in 3 of 9 Tac2-GFP neurons increased by ≥30% during CRH treatment, but these cells exhibited further increases in firing rate during the wash period and it is this wash-associated increase that accounts for an apparent statistical significance (Figure 3-6D, Table 3-4, control 0.77±0.25 Hz, CRH 0.85±0.24 Hz, wash 1.35±0.28 Hz, n=9, P>0.8 control vs CRH, P=0.001 CRH vs wash, P=0.0003 control vs wash, two-way ANOVA/Tukey). Kisspeptin neurons of both the arcuate and anteroventral periventricular nuclei have been reported to increase firing rate over time without treatment (250,251). To distinguish a technical from a biological phenomenon, we performed the same recording paradigm with ACSF vehicle. A similar pattern of a mild increase during the time corresponding to treatment followed by a continued increase during the time corresponding to wash was observed (Figure 3-6D, Table 3-4, OVX+E n=5, con 0.22±0.09 Hz, vehicle 0.27±0.14 Hz, wash 0.69±0.43 Hz, P>0.99 all comparisons; OVX n=6, con 0.84±0.28 Hz, vehicle 0.87±0.31 Hz, wash 2.05±0.82 Hz, P>0.99 control vs “treatment”, P=0.0132 control vs “wash”, p=0.0159 “treatment” vs “wash”, two-way ANOVA/Tukey). These observations suggest the increase in firing rate observed in a minority of Tac2-GFP neurons in the OVX group is a technical phenomenon.

CRH does not acutely alter firing rate of Tac2-GFP-identified cells prepared in the low E negative feedback model

Another possible caveat of the above study is that the suppressive effect of the daily surge OVX+E treatment suppressed firing activity of Tac2-GFP neurons to an extent that further acute inhibition by CRH cannot be observed. To address this, we used a low E model that mimics the diestrous state (117). The firing frequency of Tac2-GFP neurons from mice prepared in the low E model was higher those prepared in the daily surge model (Figure 3-7C). Nonetheless, CRH still had no effect on the firing activity of Tac2-GFP neurons in either OVX or OVX+E mice (Figure 3-7, Table 3-4, OVX n=8, con 1.69±0.62 Hz, CRH 2.19±0.79, wash 2.19±0.75, P=0.12 control vs CRH, two-way ANOVA/Tukey; OVX+E n=8, con 0.80±0.20 Hz, CRH 0.93±0.28 Hz, wash 1.47±0.45 Hz, P=0.87 control vs CRH, two-way ANOVA/Tukey). These results suggest that under the conditions examined, CRH does not acutely alter Tac2-GFP neuronal activity in cells from female mice.

Activating CRHR-1 increases GABAergic transmission to GnRH neurons from OVX+E mice prepared in the daily surge model

To test indirect actions of activating CRH receptors on GnRH neurons via GABAergic pathways, we examined the effect of stressin I or urocortin 3 on GABAergic PSCs in GnRH neurons in brain slices from OVX vs OVX+E mice. Figures 3-8A and 3-8C show representative traces from whole-cell voltage clamp recordings of GABAergic PSCs in

GnRH neurons from OVX and OVX+E mice before, during and after treatments. Neither stressin I (Figure 3-8A, B; n=7; control 0.51±0.14 Hz; stressin I 0.58±0.19 Hz; wash 0.57±0.16 Hz; P>0.6, Table 3-5, two-way repeated-measures ANOVA/Tukey) nor urocortin 3 (Figure 3-8C, D; n=7; control 0.52±0.13 Hz; urocortin 3 0.55±0.14 Hz; wash 0.46±0.12 Hz; P>0.7, Table 3-5, two-way repeated-measures ANOVA/Tukey) affected GABAergic PSC frequency in GnRH neurons from OVX mice. In contrast, in GnRH neurons from OVX+E mice, the CRHR-1 agonist stressin I increased GABAergic PSC frequency in 8 of 9 cells compared to the control period (Figure 3-8A, B; n=9; control 0.43±0.14 Hz; stressin I 0.71±0.23 Hz; wash 0.59±0.19 Hz; P<0.001, Table 3-5, two-way repeated-measures ANOVA/Tukey). The CRHR-2 agonist urocortin 3 did not affect GABAergic PSC frequency in GnRH neurons from OVX+E mice (Figure 3-8C, D; n=8; control 0.61±0.11 Hz; urocortin 3 0.53±0.11 Hz; wash 0.48±0.08 Hz; P>0.2, Table 5, two-way repeated-measures ANOVA/Tukey). The other PSC properties that were quantified were not altered by either stressin I or urocortin 3 during the treatment period (Figure 3-9A-C). There were no differences in passive properties among groups (Table 3-6). These results suggest that activation of CRHR-1 increases the frequency of GABAergic transmission to GnRH neurons, which because of the excitatory effect of GABA on these cells (153,154) could explain the reported increase in GnRH neuron firing activity during CRHR-1 agonist treatment (238).

Discussion

Reproduction is regulated by the central nervous system via GnRH neurons, which form the final common pathway that integrates inputs and releases GnRH to regulate the

anterior pituitary's production of gonadotropins. Stress can alter reproduction at many levels, including disruption of estradiol positive feedback action. In brain slices from unstressed mice prepared in a model of estradiol positive feedback, treatment with CRH, a mediator that is released centrally during the stress response, exerts both stimulatory and inhibitory effects on GnRH neuron activity (238). Here we provide evidence that stimulatory effects of CRH receptor activation are, at least in part, mediated by increased GABAergic transmission frequency to GnRH neurons. The mechanisms for inhibition of GnRH neurons by CRH remain elusive.

These observations support and extend prior evidence that GABA is involved in mediating both CRH- and stress-induced alterations of reproductive hormones. Injections of CRH into the bed nucleus of the stria terminalis or locus coeruleus increased Fos expression in GAD67-identified GABAergic neurons in the medial preoptic area, suggesting increased cellular activity of these cells (165,252). Administration of a $GABA_A$ receptor antagonist into the preoptic region blocks stress-induced suppression of LH pulses (162). In these studies, it is not possible to know the specific cell types affected by treatments. In the present study, bath-application of a CRHR-1 agonist to brain slices increased frequency of GABAergic transmission to GnRH neurons. GABA can induce action potential firing in GnRH neurons because these cells maintain high intracellular chloride levels (153,154). The observed increase in GABA transmission could thus explain the stimulatory effect of activation of CRHR-1 on GnRH neuron firing activity in brain slices (238).

The stimulatory effect of activating CRHR-1 on GABAergic transmission to GnRH neurons is dependent upon circulating estradiol. Estradiol also influences the reproductive response to hormones of the HPA axis *in vivo*. In OVX ewes, cortisol treatment suppresses LH pulse amplitude without affecting LH pulse frequency, suggesting no effect on GnRH release frequency (74). In contrast, in ewes given follicular-phase estradiol levels, cortisol inhibits both LH pulse amplitude and frequency, and reduces GnRH pulse frequency (61,75,76). Inhibitory effects of CRH on LH pulse frequency and amplitude are also stronger in OVX+E than OVX rats (81). Consistent with these *in vivo* measures, CRH altered GnRH neuron firing rate in cells from OVX+E but not OVX mice (238). These observations likely involve an estradiol-sensitive network upstream of GnRH neurons as estrogen receptor alpha is typically not detected in these cells (253). Supporting this postulate, changing circulating levels of estradiol alters other neuromodulatory effects on GnRH neurons. For example, the enhanced excitation of GnRH neurons elicited by kisspeptin in OVX+E vs OVX mice is in part attributable to kisspeptin increasing GABAergic transmission to GnRH neurons in the former but not the latter (244,254). Estradiol also enables time-of-day shifts in GABAergic transmission to GnRH neurons (157) and induces synaptic plasticity in several brain regions, including the hypothalamus (255-257). Of interest, CRHR-1 and estrogen receptor alpha are coexpressed in non-kisspeptin neurons in the anteroventral periventricular nucleus; cells in this area are activated following stress based on phosphorylated CREB expression (151). It is important to point out that OVX models largely alter the circulating ovarian hormone levels. Steroids are also synthesized in the brain, and it is possible that local central steroids affect responses to the treatments

used (258,259). Together, these observations suggest an integral link between estradiol and GABAergic inputs to GnRH neurons that might be attributable to estradiol-induced changes in synaptic connectivity between these cell types.

The established role of CRHR-2 in inhibiting reproduction appears to utilize alternative mechanisms. Blocking CRHR-2 prevents restraint-induced suppression of LH pulses and central injection of a CRHR-2 agonist (urocortin 2) reduced LH pulse frequency in female rats (86). Similarly, activating CRHR-2 with urocortin 3 inhibits GnRH neuron firing activity in brain slices (238). The frequency of GABAergic postsynaptic currents is modified by a number of factors that influence GnRH output, including development, steroids and nutritional status, indicating these inputs are a major node for integration (156-158,260). Reduction in frequency of GABA transmission to GnRH neurons does not, however, appear to convey the inhibitory actions of CRH receptor activation within the brain slice preparation. It is possible that GABA afferents of GnRH neurons that are affected by CRH receptor activation are not included within the slice. CRH-induced changes in GABAergic transmission to GnRH-neuron afferents cannot be excluded, nor can changes in fast glutamatergic transmission. Of note, the latter is very low frequency in GnRH neurons (209,261), thus reducing excitation through this route is not a likely explanation for inhibitory actions of CRH.

Like GABAergic neurons, KNDy neurons of the hypothalamic arcuate nucleus are a major afferent regulator of episodic GnRH/LH release. These cells produce kisspeptin and neurokinin B, both of which increase GnRH neuron output (141,142,254,262). They

also produce dynorphin; activation of dynorphin receptors *in vivo* reduces LH pulse frequency (263,264). These cells could thus potentially mediate both activating and inhibitory actions of stress on the reproductive neuroendocrine system. CRH, however, did not acutely alter firing rate of KNDy neurons under either of the circulating estradiol feedback experimental conditions tested. This initially appears contrary to several studies showing that stress might act on KNDy neurons. For example, restraint, metabolic and immunological stress all decreased *Kiss1* mRNA expression in the arcuate nucleus, as did intracerebroventricular injection of CRH (65). KNDy neurons have been reported to express CRH receptors in rats (150) and stress suppressed KNDy neural activation as determined by Fos expression in both male and female mice (148,265). The effects on Fos expression were observed three hours after stress exposure thus acute vs more chronic changes in activity cannot be distinguished. Of note, Fos expression can be induced by stimuli other than neuronal firing, such as growth factors (266). One possible explanation of these observations is that the effects of stress and CRH on KNDy neurons are more targeted towards longer-term actions, such as altering the profile of peptides expressed in these cells. This could affect their secretory output independent of a change in firing rate; this postulate is supported by reports of stress altering kisspeptin expression in the arcuate nucleus (65).

In addition to indirect actions of CRH and CRHR agonists, we examined possible intrinsic targets. Voltage-gated potassium channels play a key role in regulating neuronal activity by influencing resting membrane potential and latency to spike initiation (267,268). In GnRH neurons, these currents help mediate estradiol negative

and positive feedback, with shifts in both density and voltage dependence (197,246). In the present study, the same treatment paradigms that acutely altered GnRH neuron activity (238) failed to alter the current density of either I_A or I_K in GnRH neurons. It is still possible that activation of CRH receptors might affect other potassium current properties such as the voltage-dependence of activation or inactivation (200). The voltage protocols used in the present study were intentionally abbreviated to increase the rigor of our measurements as potassium current amplitude is very sensitive to time and recording quality.

As a broader test of intrinsic changes in GnRH neurons in response to CRH, we examined excitability. Neither CRH nor a CRHR-1 agonist altered excitability of GnRH neurons in slices from OVX+E mice. Unlike CRHR-1, activation of CRHR-2 increased excitability in about one-third of GnRH neurons, but the overall excitability of the group was not different. This may represent a subpopulation that is responsive. Alternatively, activation of CRHR-2 on neuromodulatory afferents that either activate or suppress GnRH neurons may explain some of this variability. Because GnRH neurons are widely distributed, the possible afferents remaining with each slice are different. Neuronal computation of direct and indirect effects may thus result in increased excitability in some cases.

The lack of observation of consistent direct effects of CRH receptor activation for either intrinsic mechanism studied raises the question of receptor expression by GnRH neurons. We took advantage of CRHR-1 reporter mice to identify receptor-expressing

cells. No colocalization was observed between CRHR-1 and GnRH in either male and female mice, which confirms and extends a previous report in male mice (131). CRH receptor expression has been reported in about one-third of GnRH neurons (269), but identification of G-protein-coupled receptors via immunochemistry is complicated by their low expression levels. The antibody used in that study did not distinguish CRHR subtype (269). While it remains possible that the receptor detected was CRHR-2, no colocalization between GnRH and CRHR-2 detected by CRHR-2-driven cre recombinase and floxed reporter was observed in male mice (131). Another possible explanation for the negative physiologic results is that more robust activation of either CRHR subtype may have revealed a different outcome. While we cannot exclude this possibility, the agonist doses used in the present studies are both selective and effective at inducing changes in GnRH neuron firing rate in the brain slice preparation.

The present work examined four possible mechanisms to explain the effects of CRH on GnRH neuron activity. Three of these mechanisms were not supported as being involved in this acute response, but by indicating their likely lack of involvement, our studies sculpt future studies investigating other mechanisms. It is important to point out that these negative results were obtained in cells from mice with high physiologic levels of circulating estradiol, because these are the same experimental conditions and time frames under which GnRH neurons responded to CRH (238). The present work did identify an estradiol-dependent increase in GABA transmission as being, at least in part, responsible for the estradiol-dependent increase in GnRH neuron firing rate induced by CRHR-1 activation. The interactions among the HPG and HPA axes are complicated

and the quest for inhibitory mediators, as well as other stimulatory mediators, continues. With regard to the former, reports that stress may activate neurons expressing gonadotropin-inhibitory hormone, a neuromodulator that can inhibit GnRH neurons, bears further examination (148,171,173,265).

Table 3-1. Mean±SEM and statistical parameters for current density and whole-cell passive properties of GnRH neurons (Figure 1)

	Control	**Stressin I**	**t, df, P**
I_A density (pA/pF)	452.4±101.0	407.7±92.0	1.7, 6, 0.1
I_K density (pA/pF)	157.0±40.3	152.7±37.8	0.8, 6, 0.4
Rin (MΩ)	802±122	698±83	2.0, 6, 0.1
Capacitance (pF)	12.7±1.5	12.6±1.5	1.1, 6, 0.3
Rs (MΩ)	14.9±0.7	16.3±0.6	2.2, 6, 0.1
Ihold (pA)	-13.3±7.1	-33.9±10.7	2.2, 6, 0.1
	Control	**Urocortin 3**	**t, df, P**
IA density (pA/pF)	309.9±51.5	286.7±43.1	1.8, 6, 0.1
IK density (pA/pF)	96.1±15.9	94.5±14.9	0.5, 6, 0.6
Rin (MΩ)	867±103	799±120	1.4, 6, 0.2
Capacitance (pF)	15.2±1.2	14.8±1.0	1.0, 6, 0.3
Rs (MΩ)	14.6±0.7	14.6±0.8	0.1, 6, 0.9
Ihold (pA)	-18.0±3.9	-21.4±4.1	1.7, 6, 0.1

Table 3-2. Mean±SEM and one- or two-way-repeated-measures ANOVA statistical parameters for excitability of GnRH neurons (Figures 2, 3, 4)

#spikes per current injection	Treatment	Current steps	Interaction
CRH (Fig 2B)	F(1,6)=0.21	F(19,114)=74.26*	F(19,114)=0.37
Stressin I (Fig 3B)	F(1,6)=1.74	F(19,114)=38.52*	F(19,114)=1.77
Urocortin 3 (Fig 4B)	F(1,7)=1.09	F(19,133)=26.24*	F(19,133)=1.06

*P<0.0001

CRH (n=7)	Control	CRH	Wash	F, P (One-way RM ANOVA)
pA to first spike	15.1±1.4	16.6±1.5	17.1±2.1	0.6, 0.51
ap width (ms)	0.76±0.04	0.76±0.04	0.74±0.04	1.5, 0.26
amplitude (mV)	95.7±1.5	93.8±1.6	94.2±2.4	1.5, 0.26
threshold (mV)	-47.9±1.2	-47.7±1.2	-47.8±1.4	0.2, 0.73
rate rise (mV/ms)	582.6±23.3	543.8±28.9	541.1±40.4	2.4, 0.17
AHP amplitude (mV)	-23.4±0.7	-23.7±0.9	-23.85±0.6	0.2, 0.70
AHP time (ms)	3.9±0.2	3.6±0.2	3.5±0.3	3.6, 0.06
AHP min (mV)	-71.4±1.4	-71.4±1.6	-71.6±1.1	0.1, 0.86

Stressin I (n=7)	Control	Stressin I	Wash	F, P (One-way RM ANOVA)
pA to first spike	17.1±2.0	18.0±2.4	21.1±2.1	2.9, 0.13
ap width (ms)	0.76±0.05	0.78±0.04	0.76±0.04	0.7, 0.46
amplitude (mV)	97.9±3.0	96.2±2.3	95.9±1.9	0.5, 0.53
threshold (mV)	-44.2±1.3	-43.8±1.3	-43.7±1.5	0.5, 0.60
rate rise (mV/ms)	554.0±18.7	557.5±15.1	536.9±16.9	2.2, 0.16
AHP amplitude (mV)	-25.4±0.4	-25.6±0.8	-24.6±0.9	0.9, 0.42
AHP time (ms)	4.0±0.3	4.1±0.3	3.7±0.4	2.3, 0.15
AHP min (mV)	-69.6±1.4	-69.3±1.5	-68.2±1.4	2.3, 0.16

Urocortin 3 (n=8)	Control	Urocortin 3	Wash	F, P (One-way RM ANOVA)
pA to first spike	21.3±2.8	19.3±3.5	14.3±3.9	2.4, 0.15
ap width (ms)	0.86±0.05	0.85±0.05	0.84±0.05	0.7, 0.52
amplitude (mV)	89.8±2.1	89.3±1.7	89.5±2.6	0.1, 0.83
threshold (mV)	-46.1±1.2	-47.7±1.6	-49.0±2.1	3.2, 0.10
rate rise (mV/ms)	463.2±38.6	449.5±36.5	461.0±44.8	0.3, 0.72
AHP amplitude (mV)	-24.9±1.1	-24.8±1.0	-24.5±1.2	0.2, 0.70
AHP time (ms)	4.0±0.4	3.8±0.4	3.9±0.3	0.9, 0.40
AHP min (mV)	-70.9±1.7	-72.4±1.7	-73.5±1.5	3.0, 0.11

Table 3-3. Mean±SEM of GnRH neuron whole-cell passive properties and statistical parameters for excitability recordings (Figures 2,3,4)

CRH (n=7)	Control	CRH	Wash	Statistical parameters
Rin (MΩ)	841±60	715±76	689±63	P=0.11 (Friedman test)
Capacitance (pF)	15.2±1.2	15.4±0.9	15.4±1.1	F=0.3, P=0.65 (One-way RM ANOVA)
Rs (MΩ)	14.0±0.5	13.9±1.0	14.2±0.8	F=0.3, P=0.63 (One-way RM ANOVA)
Ihold (pA)	-11.1±5.8	-13.7±5.0	-15.3±5.5	F=3.9, P=0.06 (One-way RM ANOVA)
Stressin I (n=7)	**Control**	**Stressin I**	**Wash**	
Rin (MΩ)	973±75	991±76	875±91	F=1.9, P=0.19 (One-way RM ANOVA)
Capacitance (pF)	13.1±1.3	12.82±1.3	12.9±1.4	F=0.2, P=0.71 (One-way RM ANOVA)
Rs (MΩ)	14.9±0.9	15.2±0.4	17.6±0.6	F=0.8, P=0.42 (One-way RM ANOVA)
Ihold (pA)	-14.3±3.9	-16.9±3.7	-21.7±4.0	F=4.9, P=0.06 (One-way RM ANOVA)
Urocortin 3 (n=8)	**Control**	**Urocortin 3**	**Wash**	
Rin (MΩ)	814±93	816±100	915±109	P=0.79 (Friedman test)
Capacitance (pF)	12.9±1.6	12.9±1.9	12.8±1.9	F=0.05, P=0.88 (One-way RM ANOVA)
Rs (MΩ)	14.5±0.9	15.0±1.1	15.4±1.1	F=2.3, P=0.17 (One-way RM ANOVA)
Ihold (pA)	-18.6±5.6	-17.5±7.0	-17.3±5.7	F=0.2, P=0.82 (One-way RM ANOVA)

Table 3-4. Two-way, repeated-measures ANOVA statistical parameters for extracellular Tac2-GFP neuron recordings in the daily surge model (Figure 6) and low E model (Figure 7).

CRH (daily surge)	**Steroid milieu**	**Treatment**	**Interaction**
Firing frequency	F(1,14) = 10.32*	F(2,28) = 6.28*	F(2,28) = 4.40*
CRH (low E)	**Steroid milieu**	**Treatment**	**Interaction**
Firing frequency	F(1,14) = 1.55	F(2,28) = 5.60*	F(2,28) = 1.22
Vehicle	**Steroid milieu**	**Treatment**	**Interaction**
Firing frequency	F(1,9) = 2.47	F(2,18) = 5.65*	F(2,18) = 1.20

*P<0.05

Table 3-5. Statistical parameters for GABA PSC properties (Figures 8,9). All main effects of treatment other than PSC frequency during stressin I treatment were attributable to changes during only the wash period.

Stressin I	**Steroid milieu**	**Treatment**	**Interaction**
PSC frequency (Fig 7B)	F(1,14) = 0.01	F(2,28) = 6.13**	F(2,28) = 2.09
amplitude (Fig 8A)	F(1,14) = 0.57	F(2,28) = 0.18	F(2,28) = 1.48
decay time (Fig 8B)	F(1,14) = 1.26	F(2,28) = 6.92**	F(2,28) = 2.16
fwhm (Fig 8C)	F(1,14) = 0.28	F(2,28) = 9.47**	F(2,28) = 0.49
Urocortin 3	**Steroid milieu**	**Treatment**	**Interaction**
PSC frequency (Fig 7D)	F(1,13) = 0.04	F(2,26) = 4.72*	F(2,26) = 1.62
amplitude (Fig 8A)	F(1,13) = 2.09	F(2,26) = 0.33	F(2,26) = 1.80
decay time (Fig 8B)	F(1,13) = 0.58	F(2,26) = 4.29	F(2,26) = 0.54
fwhm (Fig 8C)	F(1,13) = 0.47	F(2,26) = 10.76**	F(2,26) = 0.27

*P<0.05, **P<0.01

Table 3-6. Mean±SEM of GnRH neuron whole-cell passive properties and statistical parameters for GABA PSC recordings (Figures 8,9)

Stressin I (OVX+E)	**Control**	**Treatment**	**Wash**
Rin (MΩ)	918±149	855±119	905±124
Capacitance (pF)	15.8±1.1	15.8±1.4	15.9±1.4
Rs (MΩ)	14.5±1.0	16.3±1.4	16.1±1.4
Ihold (pA)	-26.5±7.1	-29.1±8.3	-31.7±8.9
Urocortin 3 (OVX+E)	**Control**	**Treatment**	**Wash**
Rin (MΩ)	840±140	796±150	653±104
Capacitance (pF)	16.2±1.4	15.8±0.9	15.6±1.1
Rs (MΩ)	13.6±1.3	14.4±1.3	15.2±1.0
Ihold (pA)	-29.8±7.9	-49.9±21.6	-37.9±11.1
Stressin I (OVX)	**Control**	**Treatment**	**Wash**
Rin (MΩ)	1027±88	956±77	841±95
Capacitance (pF)	11.8±1.4	11.2±1.5	11.2±1.3
Rs (MΩ)	17.8±0.6	18.0±0.8	17.8±0.5
Ihold (pA)	-24.4±3.7	-26.2±4.2	-25.3±4.6
Urocortin 3 (OVX)	**Control**	**Treatment**	**Wash**
Rin (MΩ)	866±114	888±107	788±99
Capacitance (pF)	15.4±2.4	14.4±2.3	14.5±2.2
Rs (MΩ)	15.4±1.2	14.8±1.6	15.9±1.2
Ihold (pA)	-26.2±6.5	-40.6±10.8	-34.8±8.8

Stressin I	**Steroid milieu**	**Treatment**	**Interaction**
Rin (MΩ)	F(1,14) = 0.09	F(2,28) = 1.64	F(2,28) = 1.51
Capacitance (pF)	F(1,14) = 5.34	F(2,28) = 0.42	F(2,28) = 0.58
Rs (MΩ)	F(1,14) = 2.30	F(2,28) = 2.20	F(2,28) = 1.57
Ihold (pA)	F(1,14) = 0.15	F(2,28) = 1.21	F(2,28) = 0.69
Urocortin 3	**Steroid milieu**	**Treatment**	**Interaction**
Rin (MΩ)	F(1,13) = 0.27	F(2,26) = 3.58	F(2,26) = 0.50
Capacitance (pF)	F(1,13) = 0.20	F(2,26) = 3.06	F(2,26) = 0.48
Rs (MΩ)	F(1,13) = 0.32	F(2,26) = 1.67	F(2,26) = 0.82
Ihold (pA)	F(1,13) = 0.12	F(2,26) = 2.68	F(2,26) = 0.11

Figures and Legends

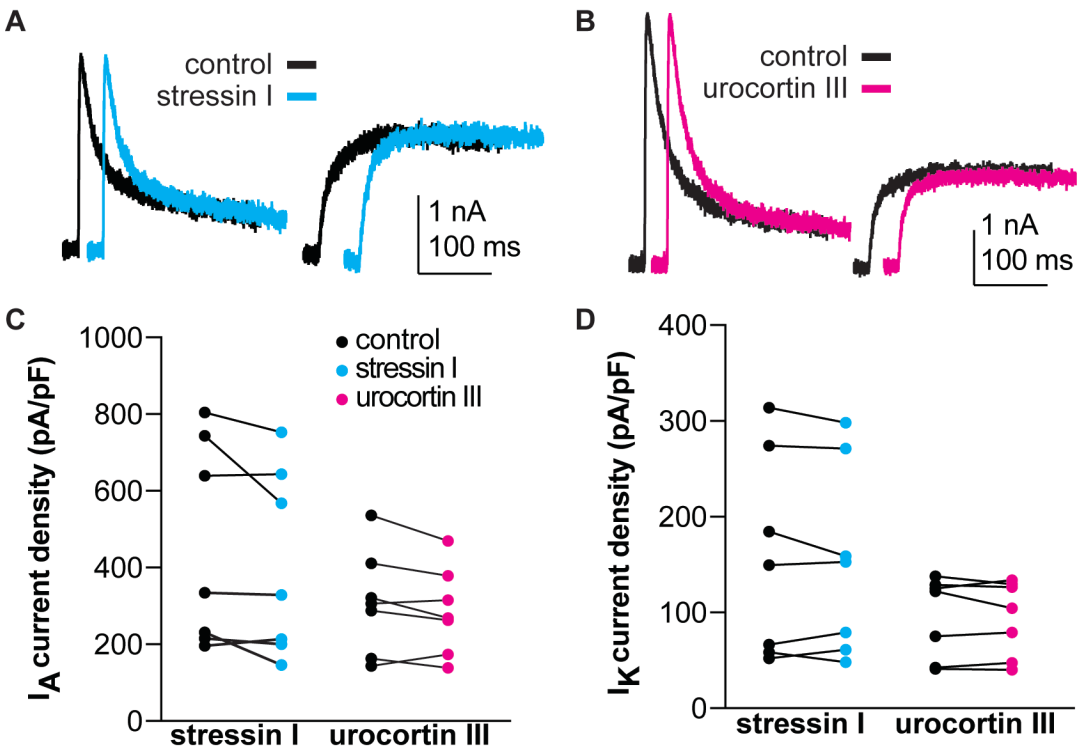

Figure 3-1. Activation of CRH receptors does not affect amplitude of voltage-gated potassium currents in GnRH neurons from OVX+E mice during positive feedback. (A-B) Representative traces of I_A and I_K during control (black) and treatment (stressin I in cyan, urocortin 3 in magenta) in GnRH neurons. (C-D) I_A or I_K current density of individual GnRH neurons during control, and stressin I or urocortin 3 treatment.

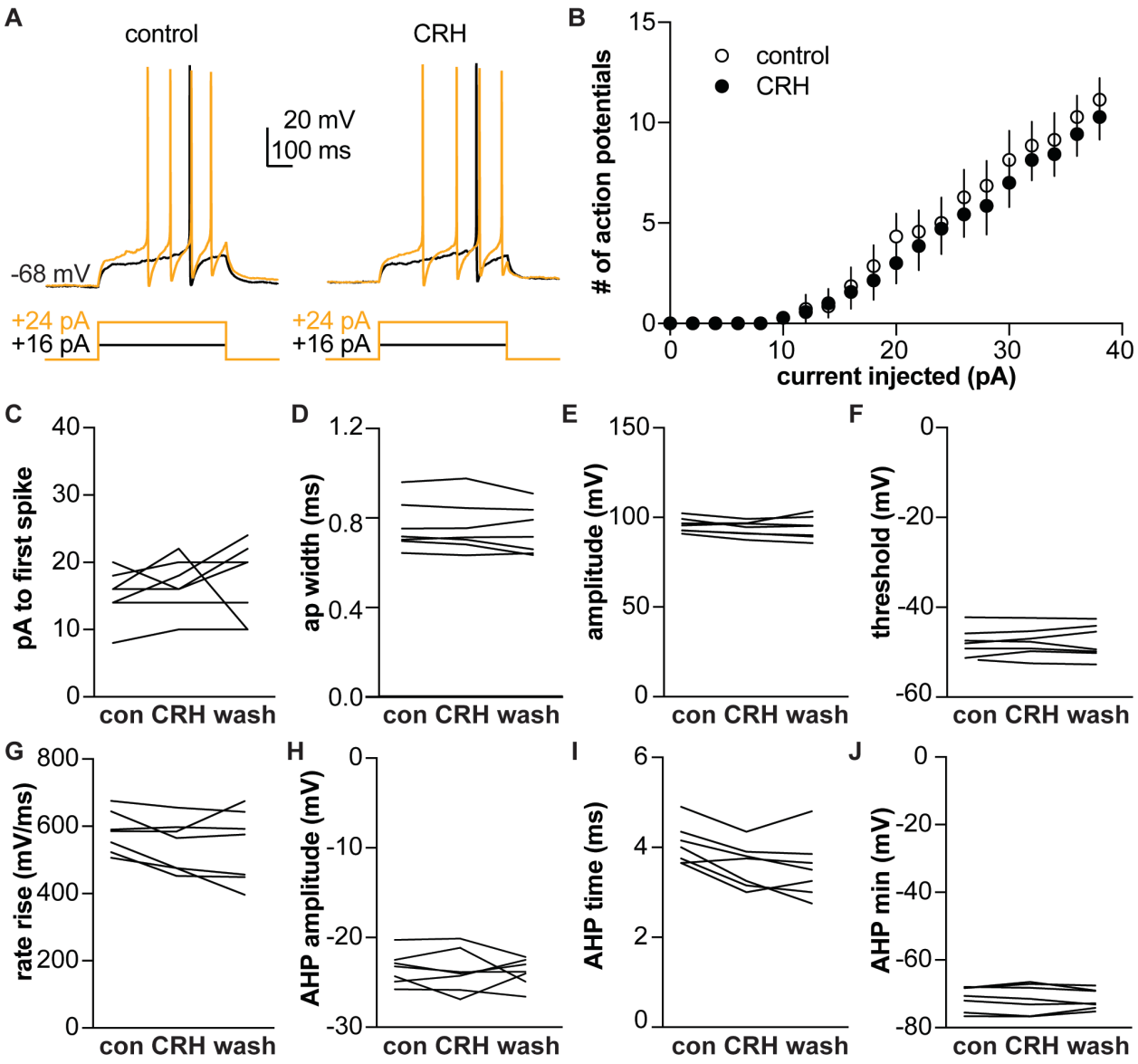

Figure 3-2. CRH does not affect GnRH neuron excitability. (A) Representative traces under control and CRH treatment conditions in OVX+E mice during positive feedback. (B) Mean±SEM number of action potentials elicited by each current injection step. (C-J) individual values during control, CRH treatment, and wash out periods. (C) Current required to generate first action potential. (D) Action potential width. (E) Action potential amplitude. (F) Action potential threshold. (G) Action potential rate of rise. (H) AHP amplitude. (I) AHP time. (J) AHP minimum.

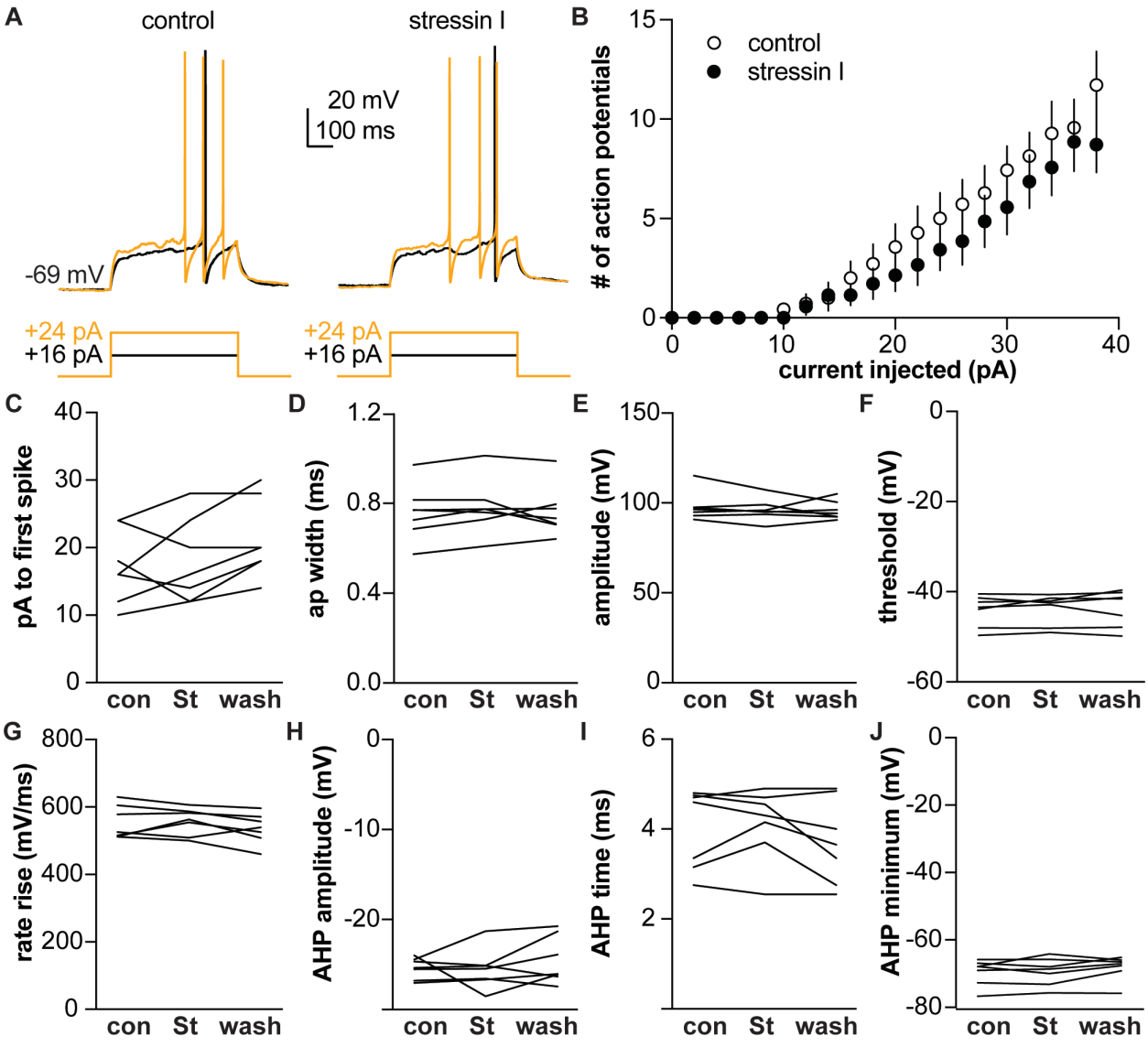

Figure 3-3. Activation of CRHR-1 with stressin I (St) does not affect GnRH neuron excitability in OVX+E mice during positive feedback. (A) Representative traces under control and stressin 1 treatment conditions. (B) Mean±SEM spikes elicited by each current injection step. (C-J) individual values during control, stressin I (St) treatment, and wash out periods. (C) Current required to generate first action potential. (D) Action potential width. (E) Action potential amplitude. (F) Action potential threshold. (G) Action potential rate of rise. (H) AHP amplitude. (I) AHP time. (J) AHP minimum.

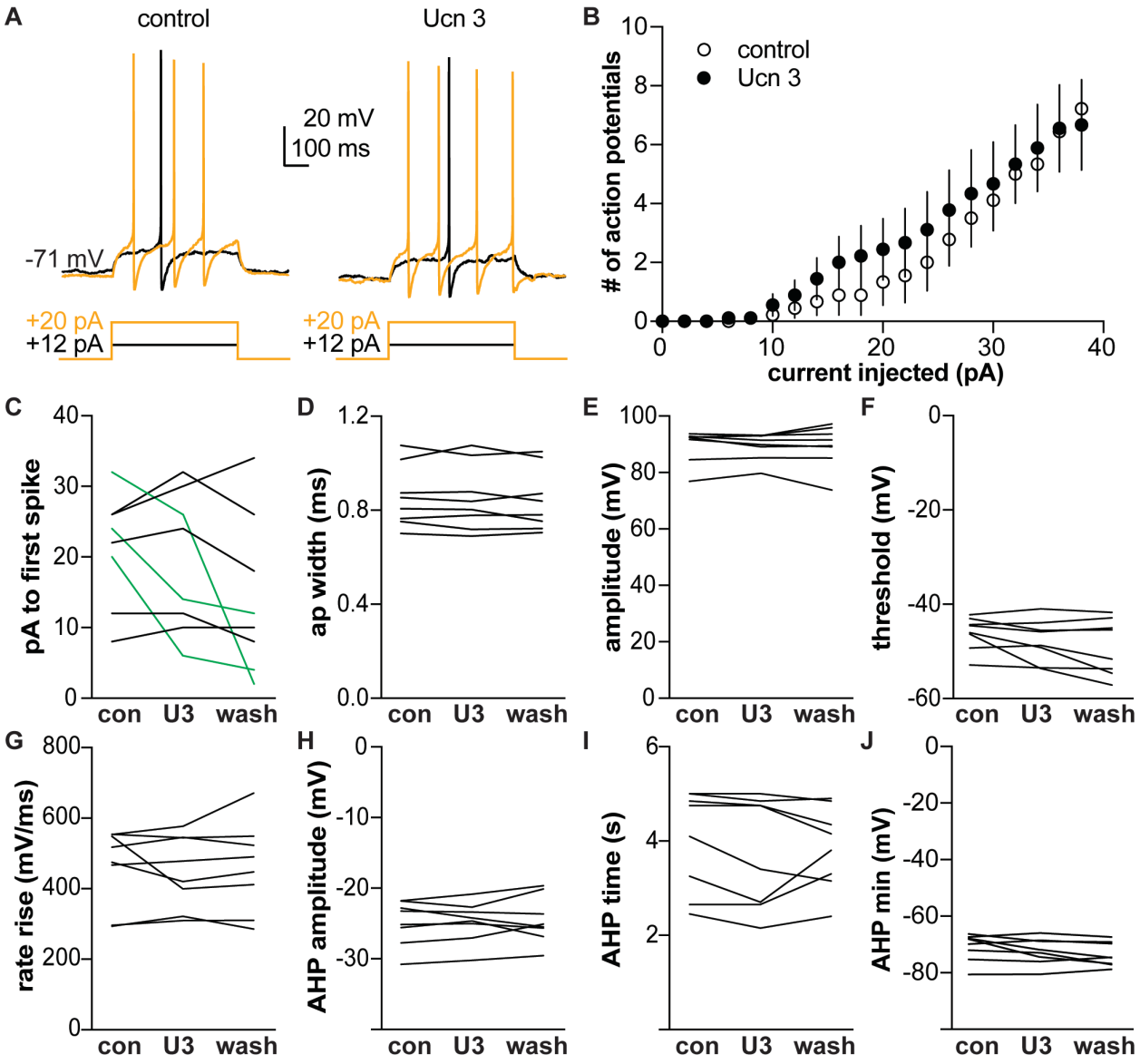

Figure 3-4. Activation of CRHR-2 with urocortin 3 (U3) does not affect GnRH neuron excitability in OVX+E mice during positive feedback. (A) Representative traces under control and urocortin 3 treatment conditions. (B) Mean±SEM spikes elicited by each current injection step. (C-J) individual values during control, urocortin 3 treatment, and wash out periods. (C) Current required to generate first action potential (rheobase). Green lines indicate cells exhibited decrease in rheobase during the treatment. (D) Action potential width. (E) Action potential amplitude. (F) Action potential threshold. (G) Action potential rate of rise. (H) AHP amplitude. (I) AHP time. (J) AHP minimum.

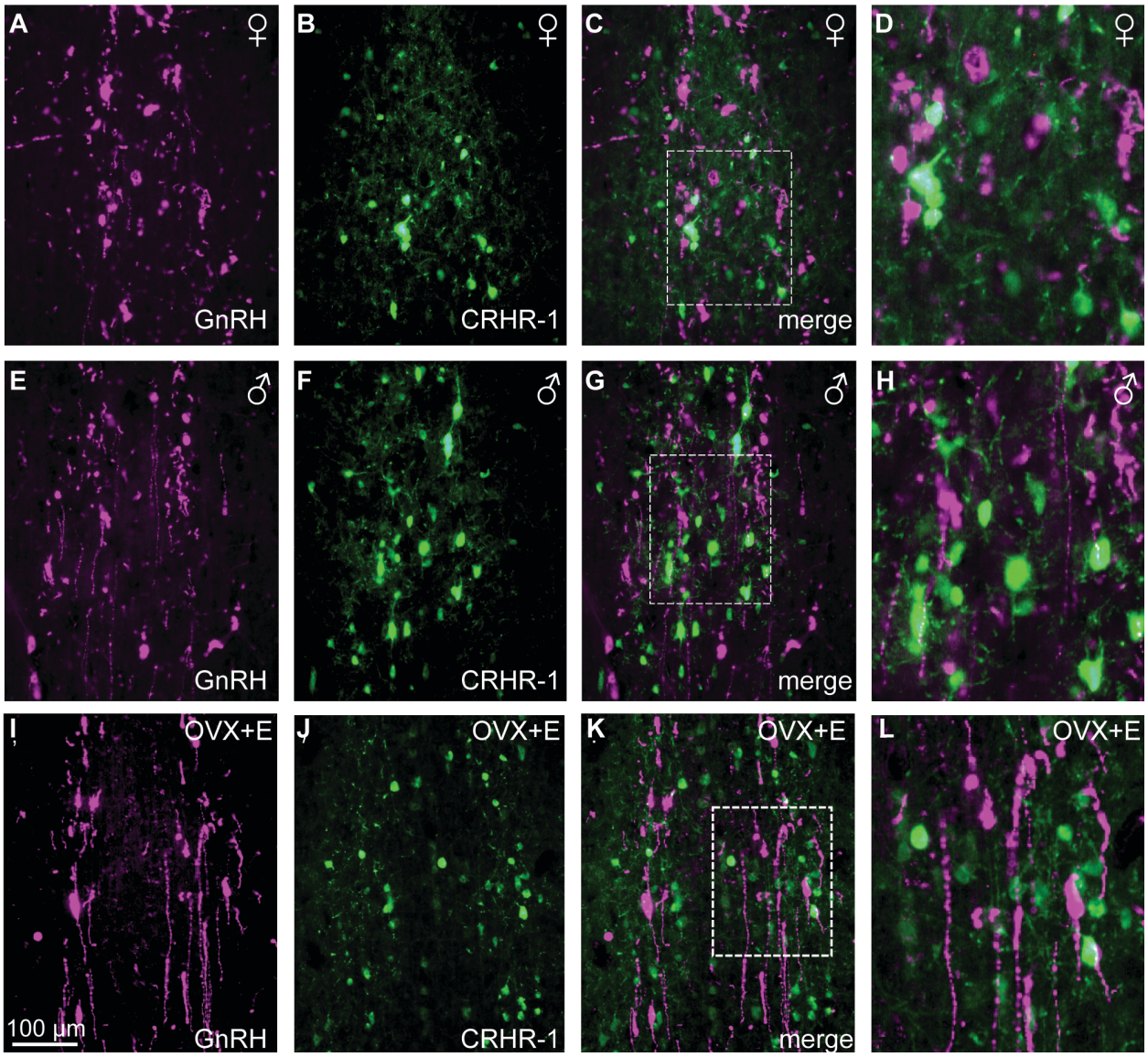

Figure 3-5. Dual-labeling of GnRH immunoreactivity and CRHR-1-GFP immunoreactivity. Representative images of GnRH and CRHR-1-GFP labeling in the preoptic area of female diestrus (A-C), male (E-G), or OVX+E female (I-K) mouse. The inset boxes (C, G and K) indicate areas further magnified in (D, H and L). No co-labeling was found although GnRH and CRHR-1-GFP neurons were often in proximity.

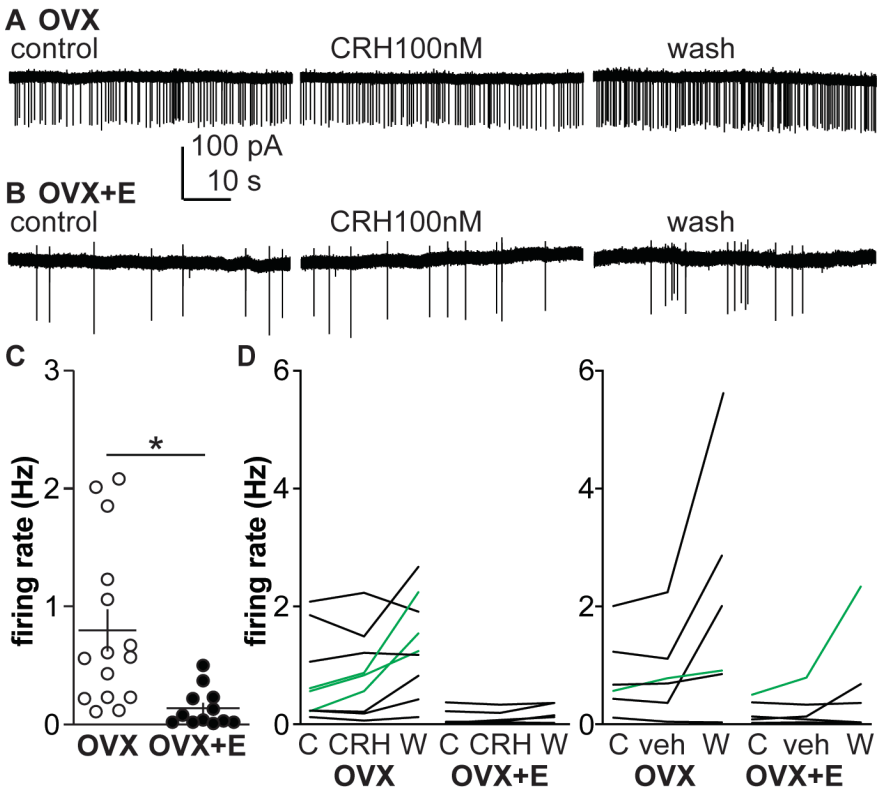

Figure 3-6. CRH does not alter firing rate of Tac2-GFP neurons from mice prepared in the daily surge positive feedback model. (A-B) Representative extracellular recordings of Tac2-GFP neurons OVX (A) and OVX+E (B) mice during each experimental period. (C) Firing rate of individual Tac2-GFP neurons from daily surge OVX and OVX+E mice during the control period. *P<0.001 two-tailed, Mann-Whitney U test. (D) Firing frequency of individual Tac2-GFP neurons during control (C), CRH (left) or vehicle (veh) (right) treatment, and wash out (W) periods. Green lines indicate cells with ≥40% increase in firing frequency during treatment, attributable to recording instability increasing firing rate during the wash period.

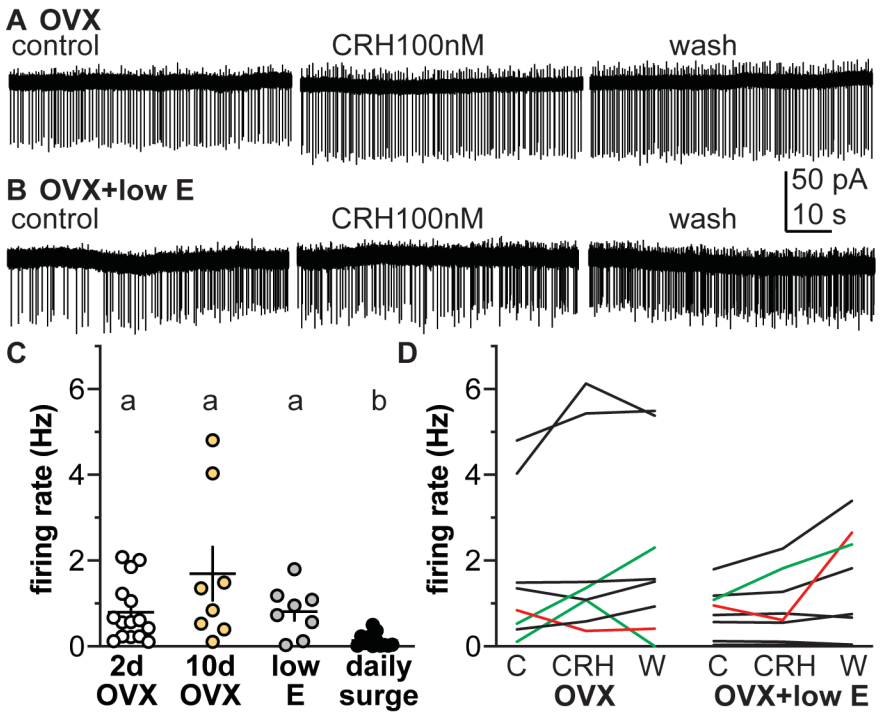

Figure 3-7. CRH does not alter firing rate of Tac2-GFP neurons from mice prepared in the low E negative feedback model of diestrus. (A-B) Representative extracellular recordings of Tac2-GFP neurons OVX (A) and OVX+ low E (B) mice during each experimental period. (C) Firing rate of individual Tac2-GFP neurons from the four groups examined (data from Figure 6C are repeated for ease of comparison); Kruskal-Wallis (KW=16.8, p~0.0008)/Dunn's post hoc * p range 0.003-0.03. (D) Firing frequency of individual Tac2-GFP neurons during control (C), CRH (left) or vehicle (veh) (right) treatment, and wash out (W) periods. Green/red lines indicate cells with ≥40% increase/decrease in firing frequency during treatment.

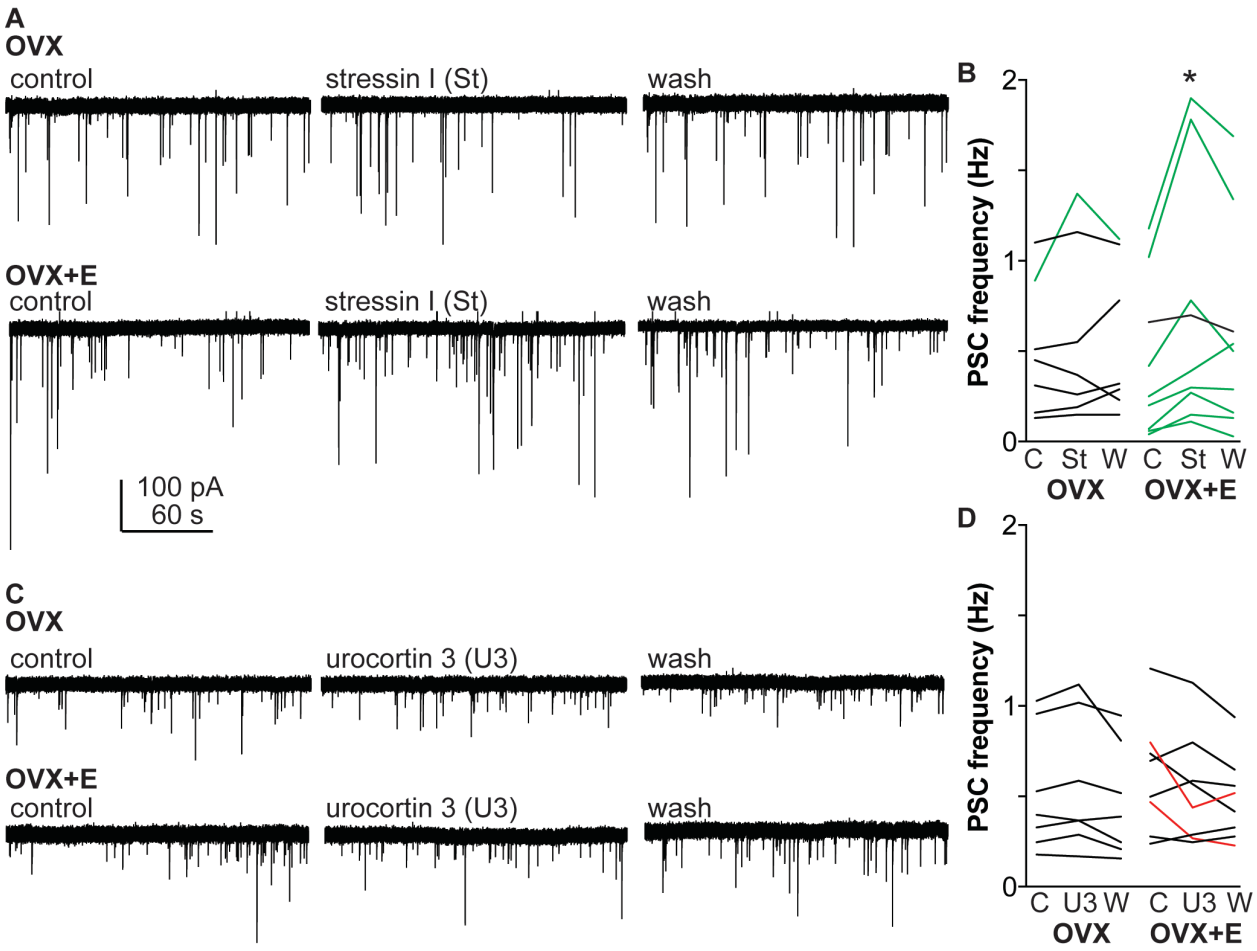

Figure 3-8. Activation of CRHR-1 with stressin I (St) but not CRHR-2 with urocortin 3 (U3) increases GABAergic transmission frequency to GnRH neurons in OVX+E mice. (A) Representative whole-cell voltage-clamp recordings of GnRH neurons treated with stressin I (St), top OVX, bottom OVX+E. (B) PSC frequency of individual GnRH neurons during control (C), stressin I, and wash out (W) periods. (C) Representative whole-cell voltage-clamp recordings of GnRH neurons treated with urocortin 3 (U3), top OVX, bottom OVX+E. (D) PSC frequency of individual GnRH neurons during control (C), urocortin 3 and wash out (W) periods. Green and red lines indicate cells with ≥30% increase or decrease in PSC frequency during treatment, respectively. *P<0.05 PSC frequency compared with control period, two-way repeated-measures ANOVA/Tukey.

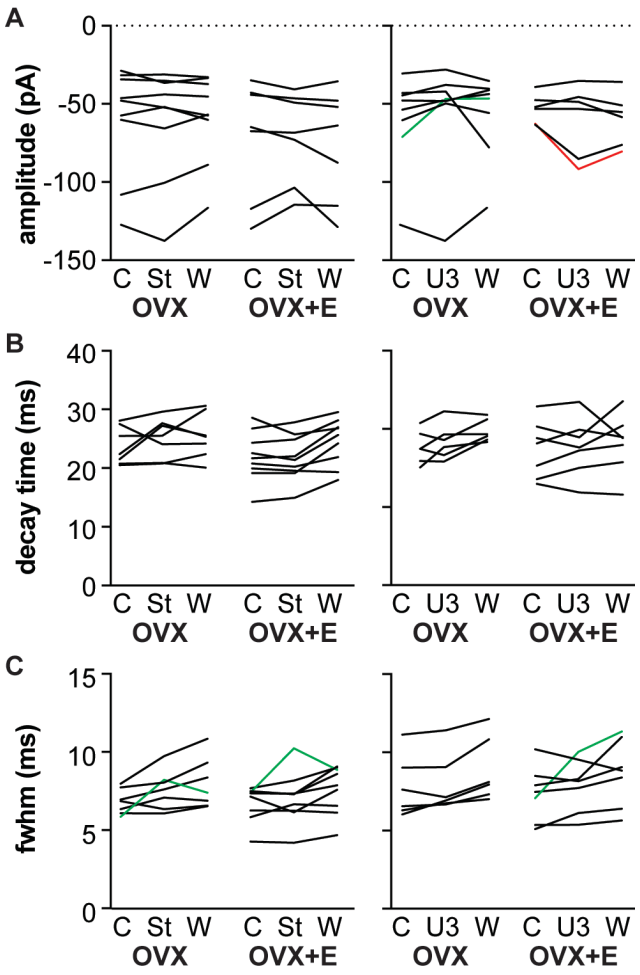

Figure 3-9. Activation of either CRHR-1 or CRHR-2 has no effect on PSC amplitude or kinetics in GnRH neurons regardless of estradiol status. (A-C) PSC properties of individual GnRH neurons during control (C), stressin I (St) or urocortin 3 (U3) treatment and wash out (W) periods. Green and red lines indicate cells with ≥30% increase or decrease in each parameter during treatment, respectively.

Chapter 4: Conclusion

This dissertation examined central mechanisms of the effect of stress by examining the action of CRH on reproduction. We observed that CRH indeed exerts effects on GnRH neuron activity as measured by the action potential firing rate of these cells in acutely-prepared brain slices. We further studied both indirect and direct neurobiological mechanisms of how CRH acts on GnRH neurons. The summary of the findings in this study is illustrated in the Figure 4-1.

Actions of CRH on GnRH neuron firing activity

CRH was postulated to be involved in stress-induced alteration of the HPG axis. In Chapter 2, effects of CRH on GnRH neuron firing activity were tested. We found that CRH could elicit both inhibitory and stimulatory effects on GnRH neurons depending on concentration of CRH and CRHR subtype activated. The direction of response to CRH was determined by the concentration of CRH, with high and low concentrations of CRH decreasing and increasing firing frequency in GnRH neurons, respectively. We postulated that the different responses we observed resulted from different subtype of CRHR activated. Using CRHR specific agonists, we found that the suppressive effect of CRH on GnRH neuron firing activity is mediated through CRHR-2. Other studies have also noted the involvement of CRHR-2 signaling on stress-induced suppression of the HPG axis. ICV injection of urocortin II, for which CRHR-2 has high affinity and CRHR-1

low affinity, suppresses LH pulse frequency and amplitude in female rats (86). Furthermore, the inhibitory effects of metabolic, psychological, and immunological stressors on LH secretion are abolished by icv injection of CRHR-2 antagonists (85). Taken together these data suggest that the inhibitory effect of stress is mediated by CRHR-2 signaling to alter GnRH neuron output, which then could affect the LH pulse secretion.

Unexpectedly, CRH also exhibited stimulatory effects on GnRH neurons via CRHR-1. This helps explain the previous studies that discovered acute stimulatory effects of stress on the reproductive system in some conditions (92,114). Proestrous rats exposed to acute restraint stress showed increased in LH and FSH secretion and blocking CRHR-1 signaling prevented the stimulatory effects of acute restraint stress on gonadotropin secretion, whereas a CRHR-2 antagonist did not alter the stimulatory effect of restraint stress (90,91). Further experiments *in vivo* are needed to test whether or not activation of CRHR-1 signaling would provide a stimulatory effect on either GnRH/LH release or the reproductive outcome. For example, one should test if activation of CRHR-1 signaling using a specific agonist would lead to an increase in LH pulse frequency or amplitude. It is important to note that the stimulatory effect of CRH on GnRH neurons does not necessary mean that stress enhances reproductive function. Continuous GnRH treatment suppresses LH and FSH secretion, whereas pulsatile treatment of GnRH maintains LH and FSH (18). Therefore, if GnRH release is constantly high due to activation by CRH, it could paradoxically lead to decrease in LH/FSH secretion and suppress the reproductive system.

The effects of CRH on GnRH neurons observed in this study are estradiol-dependent. In OVX mice, no effects of CRH on GnRH neuron activity were observed. This suggests that estradiol plays a role in enabling the response to CRH in GnRH neurons. These finding support and extend previous work showing that estradiol potentiates the effects of stress on reproduction in the whole animal level. The inhibitory effects of stress or CRH on LH pulse frequency are prominent in animals with high estradiol levels (77,110,111). The mechanism of how estradiol influences the stress response is not known yet. There are several possibilities that could underlie the effect of estradiol. For example, the expression of CRHR may be regulated by estradiol. In this regard, proestrous rats, which have highest estradiol during the estrous cycle, showed higher CRHR expression compared to diestrous rats (102). Moreover, estradiol could potentially regulate CRHR expression at transcription level because CRHR-2 promoter contains an estrogen receptor response element (104). Another possibility is that estradiol may enhance the responsiveness in GnRH neurons by modifying the synaptic interactions from upstream neurons of GnRH neurons as estradiol has been shown to affect synaptic interactions in the hypothalamus (205). These hypotheses remain to be tested to obtain more information of how the HPG axis regulates the HPA axis.

In addition to firing frequency, CRH also altered short-term burst firing pattern in GnRH neurons from OVX+E mice. Burst firing has historically been associated with hormonal secretion in neuroendocrine cells (194,222,223). Activation of CRHR-2 or high doses of CRH (100nM) led to decreases in the burst frequency in GnRH neurons from OVX+E

mice. In contrast, activation of CRHR-1 increased burst firing in GnRH neurons from OVX+E mice. In OVX mice, CRH did not affect mean firing frequency of GnRH neurons but it is possible that CRH could alter the burst firing pattern. However, neither high nor low concentrations of CRH appeared to change any of burst firing parameters in GnRH neurons from OVX mice. Taken together with the observation in GnRH neuron firing frequency, CRH has both excitatory and inhibitory on GnRH neurons both on mean firing activity and the burst firing pattern. This could eventually lead to changes in GnRH release at the terminal. The finding of bidirectional effects of CRH on GnRH neurons in this dissertation provides an explanation for the bidirectional effects of stress on reproductive system from previous studies and open possibilities for future direction in the field. For example, which endogenous ligands (CRH or urocortin family) are responsible for the inhibitory or stimulatory effects on HPG axis, does the stimulatory effect of CRH on GnRH neurons lead to increase in GnRH release from the terminal, and do inhibitory and stimulatory effects of stress happen at the same time points or different time points from a stress onset.

The mechanisms of how CRH acts on GnRH neurons

The work in the Chapter 2 shows that GnRH neuron activity is regulated by CRH. Chapter 3 examined mechanisms. There are two possible pathways that could mediate the effect of CRH on GnRH neurons, direct and indirect. First, we tested if CRH acts directly on GnRH neurons. Previous studies have shown that neuromodulators, such as kisspeptin, regulate GnRH neurons via modulation of voltage-gated potassium currents (200). In this study, two types of voltage-gated potassium currents were recorded from

GnRH neurons with CRHR-1 or CRHR-2 agonist treatment. Neither CRHR-1 or CRHR-2 agonist affected the current densities of either transient or sustained potassium currents in GnRH neurons. Although the amplitude of potassium currents was not affected by CRH treatment, it is possible that other properties such as a shift in voltage-dependent in activation or inactivation of potassium currents might be affected. Another possibility is that CRH does not modulate voltage-gated potassium currents but rather acts through other ionic conductances in GnRH neurons. We broadly explored the possible direct action of CRH by determining if the excitability of GnRH neurons is affected by CRH. Again, there was no difference in action potential generated by GnRH neurons before and during CRH treatment. The expression of CRHR by GnRH neurons is still controversial with evidence of presence and absence of expression (127,131). CRHR is a GPCR, thus the detection of CRHR expression is difficult due to the typically low expression levels of GPCRs. Our immunofluorescent data showed no colocalization observed between CRHR-1 and GnRH neuron in either female (diestrous and OVX+E) or male mice. While it remains possible that the CRHR presence in the GnRH neurons was CRHR-2, no colocalization between GnRH and CRHR-2 detected by CRHR-2-driven cre recombinase and floxed reporter was observed in male mice (131).

To explore possible indirect actions of CRH, we first tested the effect of CRH on arcuate kisspeptin (aka KNDy) neurons in the hypothalamus. KNDy neurons provide an estradiol-dependent stimulatory signal to GnRH neurons. The firing activity of KNDy neurons was not affected by CRH treatment either in the OVX or OVX+E group. Although some of the cells showed tendency to have elevated firing frequency after

CRH treatment, the vehicle treatment showed the same increase in firing rate as those observed during CRH treatment. Previous studies also noticed the increased in firing activity over time in kisspeptin neurons both in arcuate nucleus and anteriorventral periventricular nuclei (270,271). This result suggests that CRH has no acute effect on KNDy activity. One caveat in this study is that the basal firing activity of KNDy neurons in OVX+E mice is low due to the negative feedback of estradiol. If CRH exhibits inhibitory effect on KNDy neurons, it would be hard to observe a decrease in firing activity. We therefore employed an OVX+low E model in which the basal firing rate in KNDy neurons is higher than that of OVX+E daily surge model. CRH again did not acutely affect firing rate of KNDy neurons from OVX+low E mice. Another possibility is that the effect of CRH on KNDy neurons is chronic rather than acute. At whole animal level, KNDy neurons showed a reduction in neuronal activation marker (Fos) at much later time point (3h) from stress induction (148) than the acute actions we examined. Another possibility is that stress/CRH affects the peptide production in the KNDy neurons and lead to an eventual change in neuromodulator release without affecting the firing activity of these cells. Therefore, the option that KNDy neurons might be involved in CRH-induced alteration of GnRH neuron activity cannot be ruled out.

GABA is a crucial signal regulating GnRH neuron activity. In Chapter 3, results demonstrated that activation of CRHR-1 increased GABAergic PSC frequency to GnRH neurons from OVX+E mice. In contrast to majority of the brain, GABA is excitatory to GnRH neurons due to the high intracellular chloride levels in these cells (153,154). Thus, the stimulatory effect of CRHR-1 agonist on firing activity of GnRH neurons

shown in the Chapter 2 could stem at least in part from the increase in GABAergic transmission to GnRH neurons. Interestingly, CRHR-1 appears to be expressed in GABAergic neurons in preoptic area that are located near GnRH neurons (131). Further, GABAergic neurons in medial preoptic area showed increased in neuronal activation (indicated by Fos expression) after CRH injection into bed nucleus of stria terminalis or locus coeruleus (164,165). Although in our study activation of CRHR-2 did not affect GABAergic transmission to GnRH neurons, it is possible that it could alter GABAergic inputs to other afferents of GnRH neurons. GnRH neurons also receive glutamatergic input, although glutamatergic input frequency is much lower than that of GABAergic inputs (209,272). Nonetheless, there is a possibility that CRH could affect glutamatergic inputs to GnRH neurons and alter GnRH neuron activity, or use glutamatergic intermediates to activate other modulators of GnRH neurons. These findings together with our results suggest that GABAergic signaling could be the underlying pathway in stress/CRH-induced stimulation of reproduction.

Role of CRH in stress-induced alteration of HPG axis *in vivo*

The work in Chapter 2 and 3 explored the effect of CRH on GnRH neurons in various aspects in brain slices. An important future direction of this work is to study the role of CRH on HPG axis *in vivo*. One of the reproductive functions that has been shown to be interrupted by stress is the generation of LH surge (118,119). Continuous infusion of CRH or single injection of CRH before the onset of LH surge diminished LH surge amplitude in proestrous rats (123). Therefore, the stress-induced suppression of might be mediated by CRH. With the development of the chemogenetic manipulation

technique called designer receptors exclusively activated by designer drugs (DREADDs), the activity of targeted cells could be manipulated using either activating (HM3Dq) or inhibitory (HM4Di) designer receptors. To test if CRH is required for the suppression of LH surge, inhibitory DREADDs could be employed to suppress activity of CRH neurons and determine if the suppression effect of stress on LH surge is affected. Traditionally, the administration route for CNO (a ligand for DREADD receptor) is through intraperitoneal (ip) injection. However, data from my pilot studies with others in the lab show that ip injection could induce stress by raising corticosterone secretion in mice. Therefore, an alternative method to administer CNO that is less stressful is needed.

The alternative approach that we tested is to use Nutella to deliver the CNO to mice. This method has been shown to be an effective way to administer estradiol in mice and rats (273,274). To verify if the Nutella method is capable of delivering CNO, mice with 3Dq receptors expressing in CRH neurons (CRH-3Dq) were used. Mice were treated with Nutella-CNO following by serial blood samples for corticosterone measurement as an output of CRH neuron activity. Figure 4-2A showed that the corticosterone level in CNO-treated mice was higher than that of vehicle group but start to decline 1.5h after the treatment. In the vehicle-treated mice, the corticosterone level started to increase later in the day due to the diurnal rise of corticosterone, while the CNO-treated group did not show the diurnal increase of corticosterone. This result implies the negative feedback effect of corticosterone on CRH neurons in the CNO-treated group. This idea is supported by another set of experiment in CRH-3Dq mice treated by ip injection

method. Figure 4-2B showed that mice treated with CNO also did not show the diurnal increase in corticosterone. We further verified the Nutella-CNO method comparing to the traditional ip injection route in GnRH-3Dq mice and measured LH levels as an output of the system. Figure 4-3 showed that mice treated with CNO either with Nutella or ip injection method showed a similar increase in LH level 15min after the treatment. These results suggest that Nutella method is effective for delivering CNO and that the onset of actions can be rapid. Some modifications are still needed to solve the negative feedback issue and stress induced by a novel object during the Nutella treatment. After fine tuning the Nutella method, future experiment would use this approach with the CRH-4Di mice to suppress activity of CRH neurons and test if CRH is required for the suppression of LH surge.

Summary

The work in this dissertation provides increased understanding of the central mechanisms of how CRH interacts with the HPG axis in females. The discovery of both stimulatory and inhibitory effects of CRH on GnRH neurons are novel keys to look at the interaction between HPA and HPG axes in a new perspective. In the past, the stimulatory effects of stress are less well-known and were sometime are ignored. This work helps guide future research to explore the stimulatory effects of stress on reproductive system in more detail. The inhibitory pathways of CRH still remain to be explored to build a complete picture of how stress affects reproduction.

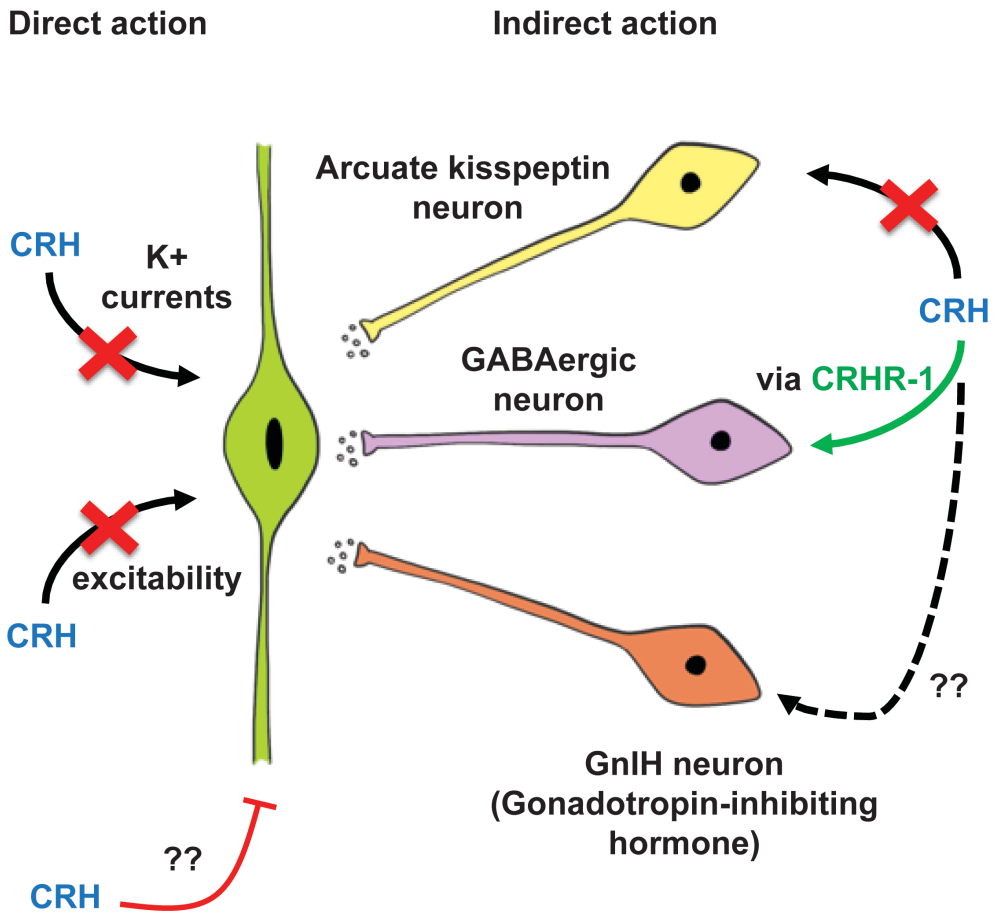

Figure 4-1. Schematic model of the actions of CRH on GnRH neurons.

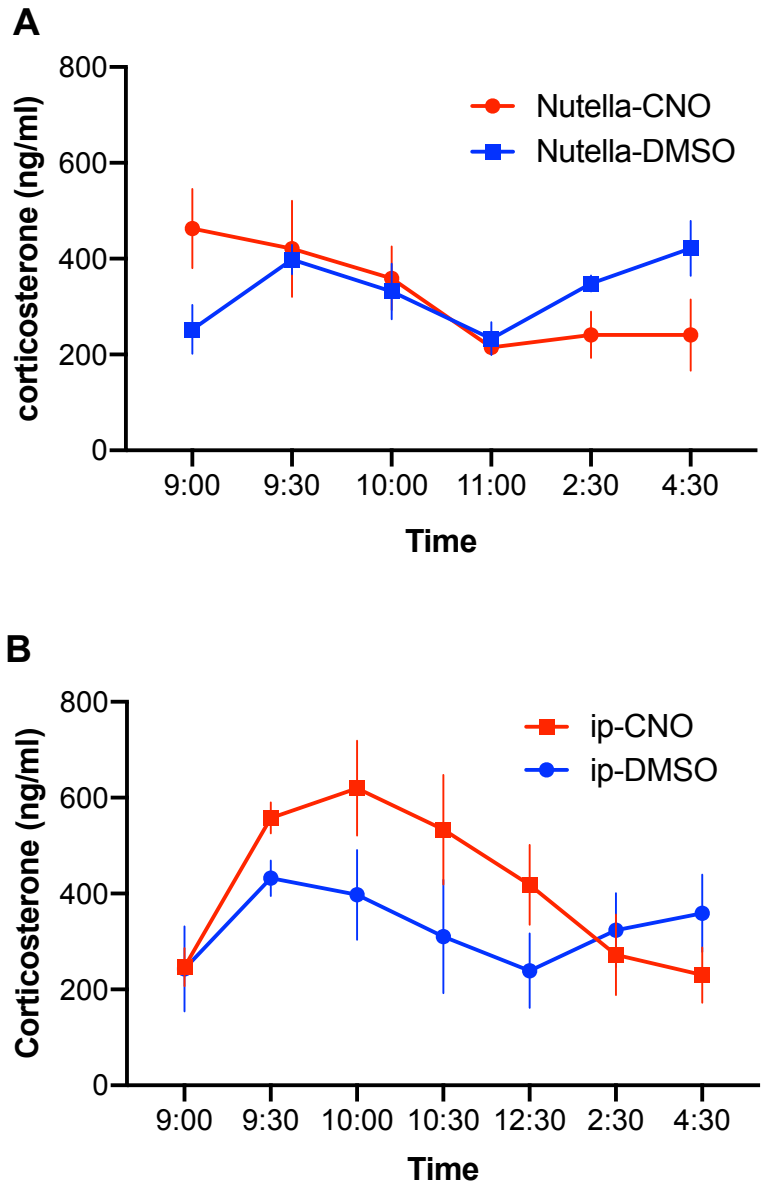

Figure 4-2. Corticosterone levels in CRH-3Dq mice treated with either CNO or DMSO via the Nutella method (A) or intraperitoneal injection (B).

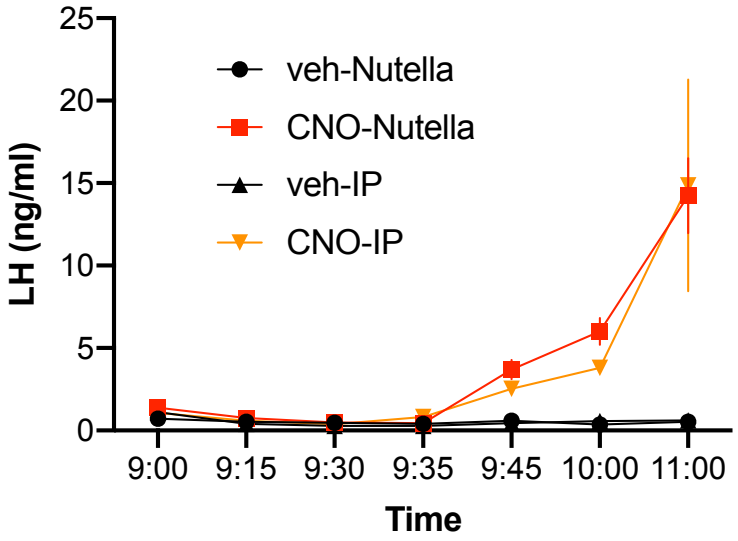

Figure 4-3. LH levels in GnRH-3Dq mice treated with either CNO or DMSO via the Nutella method or intraperitoneal injection.

Bibliography

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